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- (71) Applicant (*for all designated States except US*): **EX-ELIXIS, INC.** [US/US]; P.O. Box 511, 170 Harbor Way, South San Francisco, CA 94083-0511 (US).
- (72) Inventors; and
- (75) Inventors/Applicants (*for US only*): **GENDREAU, Steven, Brian** [US/US]; 2801 Turk Street, #103, San Francisco, CA 94118 (US). **DORA, Emery, G. III** [US/US]; 1847 32nd Avenue, San Francisco, CA 94122 (US). **LICKTEIG, Kim** [US/US]; 1921 Grove Street, San Francisco, CA 94117 (US). **AMUNDSEN, Craig, D.** [US/US]; 31 Alviso Court, Pacifica, CA 94044 (US).
- (74) Agents: **SHAYESTEH, Laleh** et al.; Exelixis, Inc., P.O. Box 511, 170 Harbor Way, South San Francisco, CA 94083-0511 (US).
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(54) Title: MAXS AS MODIFIERS OF THE AXIN PATHWAY AND METHODS OF USE

(57) Abstract: Human MAX genes are identified as modulators of the AXIN pathway, and thus are therapeutic targets for disorders associated with defective AXIN function. Methods for identifying modulators of AXIN, comprising screening for agents that modulate the activity of MAX are provided.



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MAXS AS MODIFIERS OF THE AXIN PATHWAY AND METHODS OF USE

REFERENCE TO RELATED APPLICATIONS

This application claims priority to U.S. provisional patent applications 60/401,534
5 filed 8/6/2002, and 60/411,153 filed 9/16/2002. The contents of the prior applications are
hereby incorporated in their entirety.

BACKGROUND OF THE INVENTION

Deregulation of beta-catenin signaling is a frequent and early event in the
10 development of a variety of human tumors, including colon cancer, melanoma, ovarian
cancer, and prostate cancer. Activation of beta-catenin signaling can occur in tumor cells
by loss-of-function mutations in the tumor suppressor genes Axin or APC, as well as by
gain-of-function mutations in the oncogene beta-catenin itself. Axin normally functions as
a scaffolding protein that binds beta-catenin, APC, and the serine/threonine kinase GSK3-
15 beta. Assembly of this degradation complex allows GSK3-beta to phosphorylate beta-
catenin, which leads to beta-catenin ubiquitination and degradation by the proteasome. In
the absence of Axin activity, beta-catenin protein becomes stabilized and accumulates in
the nucleus where it acts as a transcriptional co-activator with TCF for the induction of
target genes, including the cell cycle regulators cyclin D1 and c-Myc.

20 The *C. elegans* gene *pry-1* is the structural and functional ortholog of vertebrate
Axin (Korswagen HC et al. (2002) Genes Dev. 16:1291-302). PRY-1 is predicted to
contain conserved RGS and DIX domains that, in Axin, bind APC and Dishevelled,
respectively. Overexpression of the *C. elegans pry-1* gene in zebrafish can fully rescue
the mutant phenotype of *masterblind*, the zebrafish Axin1 mutation. *pry-1* loss-of-
25 function mutations produce several phenotypes that appear to result from increased beta-
catenin signaling (Gleason JE et al. (2002) Genes Dev. 16:1281-90; Korswagen et al.,
supra).

The ability to manipulate the genomes of model organisms such as *C. elegans*
provides a powerful means to analyze biochemical processes that, due to significant
30 evolutionary conservation, have direct relevance to more complex vertebrate organisms.
Due to a high level of gene and pathway conservation, the strong similarity of cellular
processes, and the functional conservation of genes between these model organisms and
mammals, identification of the involvement of novel genes in particular pathways and
their functions in such model organisms can directly contribute to the understanding of the

correlative pathways and methods of modulating them in mammals (see, for example, Dulubova I, et al, J Neurochem 2001 Apr;77(1):229-38; Cai T, et al., Diabetologia 2001 Jan;44(1):81-8; Pasquinelli AE, et al., Nature. 2000 Nov 2;408(6808):37-8; Ivanov IP, et al., EMBO J 2000 Apr 17;19(8):1907-17; Vajo Z et al., Mamm Genome 1999

5 Oct;10(10):1000-4). For example, a genetic screen can be carried out in an invertebrate model organism having underexpression (e.g. knockout) or overexpression of a gene (referred to as a “genetic entry point”) that yields a visible phenotype. Additional genes are mutated in a random or targeted manner. When a gene mutation changes the original phenotype caused by the mutation in the genetic entry point, the gene is identified as a
10 “modifier” involved in the same or overlapping pathway as the genetic entry point. When the genetic entry point is an ortholog of a human gene implicated in a disease pathway, such as AXIN, modifier genes can be identified that may be attractive candidate targets for novel therapeutics.

All references cited herein, including patents, patent applications, publications, and
15 sequence information in referenced Genbank identifier numbers, are incorporated herein in their entireties.

SUMMARY OF THE INVENTION

We have discovered genes that modify the AXIN pathway in *C. elegans* and, and
20 identified their human orthologs, hereinafter referred to as modifier of AXIN (MAX). The invention provides methods for utilizing these AXIN modifier genes and polypeptides to identify MAX-modulating agents that are candidate therapeutic agents that can be used in the treatment of disorders associated with defective or impaired AXIN function and/or MAX function. Preferred MAX-modulating agents specifically bind to MAX
25 polypeptides and restore AXIN function. Other preferred MAX-modulating agents are nucleic acid modulators such as antisense oligomers and RNAi that repress MAX gene expression or product activity by, for example, binding to and inhibiting the respective nucleic acid (i.e. DNA or mRNA).

MAX modulating agents may be evaluated by any convenient *in vitro* or *in vivo*
30 assay for molecular interaction with a MAX polypeptide or nucleic acid. In one embodiment, candidate MAX modulating agents are tested with an assay system comprising a MAX polypeptide or nucleic acid. Agents that produce a change in the activity of the assay system relative to controls are identified as candidate AXIN modulating agents. The assay system may be cell-based or cell-free. MAX-modulating

agents include MAX related proteins (e.g. dominant negative mutants, and biotherapeutics); MAX -specific antibodies; MAX -specific antisense oligomers and other nucleic acid modulators; and chemical agents that specifically bind to or interact with MAX or compete with MAX binding partner (e.g. by binding to a MAX binding partner).

- 5 In one specific embodiment, a small molecule modulator is identified using a binding assay. In specific embodiments, the screening assay system is selected from an apoptosis assay, a cell proliferation assay, an angiogenesis assay, and a hypoxic induction assay.

- 10 In another embodiment, candidate AXIN pathway modulating agents are further tested using a second assay system that detects changes in the AXIN pathway, such as angiogenic, apoptotic, or cell proliferation changes produced by the originally identified candidate agent or an agent derived from the original agent. The second assay system may use cultured cells or non-human animals. In specific embodiments, the secondary assay system uses non-human animals, including animals predetermined to have a disease or disorder implicating the AXIN pathway, such as an angiogenic, apoptotic, or cell
- 15 proliferation disorder (e.g. cancer).

- The invention further provides methods for modulating the MAX function and/or the AXIN pathway in a mammalian cell by contacting the mammalian cell with an agent that specifically binds a MAX polypeptide or nucleic acid. The agent may be a small molecule modulator, a nucleic acid modulator, or an antibody and may be administered to
- 20 a mammalian animal predetermined to have a pathology associated the AXIN pathway.

DETAILED DESCRIPTION OF THE INVENTION

- Genetic screens were designed to identify modifiers of the axin pathway in *C. elegans*, where a reduction of function *pry-1* (*axin*) mutant was used. Various specific
- 25 genes were silenced by RNA inhibition (RNAi). Methods for using RNAi to silence genes in *C. elegans* are known in the art (Fire A, et al., 1998 *Nature* 391:806-811; Fire, A. *Trends Genet.* 15, 358-363 (1999); WO9932619). Genes causing altered phenotypes in the worms were identified as modifiers of the AXIN pathway, followed by identification of their orthologs. Accordingly, vertebrate orthologs of these modifiers, and preferably
- 30 the human orthologs, MAX genes (i.e., nucleic acids and polypeptides) are attractive drug targets for the treatment of pathologies associated with a defective AXIN signaling pathway, such as cancer. Table 1 (Example II) lists the modifiers and their orthologs.

In vitro and in vivo methods of assessing MAX function are provided herein. Modulation of the MAX or their respective binding partners is useful for understanding

the association of the AXIN pathway and its members in normal and disease conditions and for developing diagnostics and therapeutic modalities for AXIN related pathologies. MAX-modulating agents that act by inhibiting or enhancing MAX expression, directly or indirectly, for example, by affecting a MAX function such as enzymatic (e.g., catalytic) or binding activity, can be identified using methods provided herein. MAX modulating agents are useful in diagnosis, therapy and pharmaceutical development.

Nucleic acids and polypeptides of the invention

Sequences related to MAX nucleic acids and polypeptides that can be used in the invention are disclosed in Genbank (referenced by Genbank identifier (GI) or RefSeq number), shown in Table 1.

The term "MAX polypeptide" refers to a full-length MAX protein or a functionally active fragment or derivative thereof. A "functionally active" MAX fragment or derivative exhibits one or more functional activities associated with a full-length, wild-type MAX protein, such as antigenic or immunogenic activity, enzymatic activity, ability to bind natural cellular substrates, etc. The functional activity of MAX proteins, derivatives and fragments can be assayed by various methods known to one skilled in the art (Current Protocols in Protein Science (1998) Coligan *et al.*, eds., John Wiley & Sons, Inc., Somerset, New Jersey) and as further discussed below. In one embodiment, a functionally active MAX polypeptide is a MAX derivative capable of rescuing defective endogenous MAX activity, such as in cell based or animal assays; the rescuing derivative may be from the same or a different species. For purposes herein, functionally active fragments also include those fragments that comprise one or more structural domains of a MAX, such as a kinase domain or a binding domain. Protein domains can be identified using the PFAM program (Bateman A., et al., Nucleic Acids Res, 1999, 27:260-2). Methods for obtaining MAX polypeptides are also further described below. In some embodiments, preferred fragments are functionally active, domain-containing fragments comprising at least 25 contiguous amino acids, preferably at least 50, more preferably 75, and most preferably at least 100 contiguous amino acids of a MAX. In further preferred embodiments, the fragment comprises the entire functionally active domain.

The term "MAX nucleic acid" refers to a DNA or RNA molecule that encodes a MAX polypeptide. Preferably, the MAX polypeptide or nucleic acid or fragment thereof is from a human, but can also be an ortholog, or derivative thereof with at least 70% sequence identity, preferably at least 80%, more preferably 85%, still more preferably

90%, and most preferably at least 95% sequence identity with human MAX. Methods of identifying orthologs are known in the art. Normally, orthologs in different species retain the same function, due to presence of one or more protein motifs and/or 3-dimensional structures. Orthologs are generally identified by sequence homology analysis, such as

5 BLAST analysis, usually using protein bait sequences. Sequences are assigned as a potential ortholog if the best hit sequence from the forward BLAST result retrieves the original query sequence in the reverse BLAST (Huynen MA and Bork P, Proc Natl Acad Sci (1998) 95:5849-5856; Huynen MA *et al.*, Genome Research (2000) 10:1204-1210). Programs for multiple sequence alignment, such as CLUSTAL (Thompson JD et al, 1994,

10 Nucleic Acids Res 22:4673-4680) may be used to highlight conserved regions and/or residues of orthologous proteins and to generate phylogenetic trees. In a phylogenetic tree representing multiple homologous sequences from diverse species (e.g., retrieved through BLAST analysis), orthologous sequences from two species generally appear closest on the tree with respect to all other sequences from these two species. Structural threading or

15 other analysis of protein folding (e.g., using software by ProCeryon, Biosciences, Salzburg, Austria) may also identify potential orthologs. In evolution, when a gene duplication event follows speciation, a single gene in one species, such as *C.elegans*, may correspond to multiple genes (paralogs) in another, such as human. As used herein, the term "orthologs" encompasses paralogs. As used herein, "percent (%) sequence identity"

20 with respect to a subject sequence, or a specified portion of a subject sequence, is defined as the percentage of nucleotides or amino acids in the candidate derivative sequence identical with the nucleotides or amino acids in the subject sequence (or specified portion thereof), after aligning the sequences and introducing gaps, if necessary to achieve the maximum percent sequence identity, as generated by the program WU-BLAST-2.0a19

25 (Altschul *et al.*, J. Mol. Biol. (1997) 215:403-410) with all the search parameters set to default values. The HSP S and HSP S2 parameters are dynamic values and are established by the program itself depending upon the composition of the particular sequence and composition of the particular database against which the sequence of interest is being searched. A % identity value is determined by the number of matching identical

30 nucleotides or amino acids divided by the sequence length for which the percent identity is being reported. "Percent (%) amino acid sequence similarity" is determined by doing the same calculation as for determining % amino acid sequence identity, but including conservative amino acid substitutions in addition to identical amino acids in the computation.

A conservative amino acid substitution is one in which an amino acid is substituted for another amino acid having similar properties such that the folding or activity of the protein is not significantly affected. Aromatic amino acids that can be substituted for each other are phenylalanine, tryptophan, and tyrosine; interchangeable hydrophobic amino acids are leucine, isoleucine, methionine, and valine; interchangeable polar amino acids are glutamine and asparagine; interchangeable basic amino acids are arginine, lysine and histidine; interchangeable acidic amino acids are aspartic acid and glutamic acid; and interchangeable small amino acids are alanine, serine, threonine, cysteine and glycine.

Alternatively, an alignment for nucleic acid sequences is provided by the local homology algorithm of Smith and Waterman (Smith and Waterman, 1981, *Advances in Applied Mathematics* 2:482-489; database: European Bioinformatics Institute; Smith and Waterman, 1981, *J. of Molec.Biol.*, 147:195-197; Nicholas et al., 1998, "A Tutorial on Searching Sequence Databases and Sequence Scoring Methods" (www.psc.edu) and references cited therein.; W.R. Pearson, 1991, *Genomics* 11:635-650). This algorithm can be applied to amino acid sequences by using the scoring matrix developed by Dayhoff (Dayhoff: *Atlas of Protein Sequences and Structure*, M. O. Dayhoff ed., 5 suppl. 3:353-358, National Biomedical Research Foundation, Washington, D.C., USA), and normalized by Gribskov (Gribskov 1986 *Nucl. Acids Res.* 14(6):6745-6763). The Smith-Waterman algorithm may be employed where default parameters are used for scoring (for example, gap open penalty of 12, gap extension penalty of two). From the data generated, the "Match" value reflects "sequence identity."

Derivative nucleic acid molecules of the subject nucleic acid molecules include sequences that hybridize to the nucleic acid sequence of a MAX. The stringency of hybridization can be controlled by temperature, ionic strength, pH, and the presence of denaturing agents such as formamide during hybridization and washing. Conditions routinely used are set out in readily available procedure texts (*e.g.*, *Current Protocol in Molecular Biology*, Vol. 1, Chap. 2.10, John Wiley & Sons, Publishers (1994); Sambrook *et al.*, *Molecular Cloning*, Cold Spring Harbor (1989)). In some embodiments, a nucleic acid molecule of the invention is capable of hybridizing to a nucleic acid molecule containing the nucleotide sequence of a MAX under high stringency hybridization conditions that are: prehybridization of filters containing nucleic acid for 8 hours to overnight at 65° C in a solution comprising 6X single strength citrate (SSC) (1X SSC is 0.15 M NaCl, 0.015 M Na citrate; pH 7.0), 5X Denhardt's solution, 0.05% sodium pyrophosphate and 100 µg/ml herring sperm DNA; hybridization for 18-20 hours at 65° C

in a solution containing 6X SSC, 1X Denhardt's solution, 100 μ g/ml yeast tRNA and 0.05% sodium pyrophosphate; and washing of filters at 65° C for 1h in a solution containing 0.1X SSC and 0.1% SDS (sodium dodecyl sulfate).

5 In other embodiments, moderately stringent hybridization conditions are used that are: pretreatment of filters containing nucleic acid for 6 h at 40° C in a solution containing 35% formamide, 5X SSC, 50 mM Tris-HCl (pH7.5), 5mM EDTA, 0.1% PVP, 0.1% Ficoll, 1% BSA, and 500 μ g/ml denatured salmon sperm DNA; hybridization for 18-20h at 40° C in a solution containing 35% formamide, 5X SSC, 50 mM Tris-HCl (pH7.5), 5mM EDTA, 0.02% PVP, 0.02% Ficoll, 0.2% BSA, 100 μ g/ml salmon sperm DNA, and 10% (wt/vol) dextran sulfate; followed by washing twice for 1 hour at 55° C in a solution containing 2X SSC and 0.1% SDS.

Alternatively, low stringency conditions can be used that are: incubation for 8 hours to overnight at 37° C in a solution comprising 20% formamide, 5 x SSC, 50 mM sodium phosphate (pH 7.6), 5X Denhardt's solution, 10% dextran sulfate, and 20 μ g/ml 15 denatured sheared salmon sperm DNA; hybridization in the same buffer for 18 to 20 hours; and washing of filters in 1 x SSC at about 37° C for 1 hour.

Isolation, Production, Expression, and Mis-expression of MAX Nucleic Acids and Polypeptides

20 MAX nucleic acids and polypeptides, are useful for identifying and testing agents that modulate MAX function and for other applications related to the involvement of MAX in the AXIN pathway. MAX nucleic acids and derivatives and orthologs thereof may be obtained using any available method. For instance, techniques for isolating cDNA or genomic DNA sequences of interest by screening DNA libraries or by using polymerase 25 chain reaction (PCR) are well known in the art. In general, the particular use for the protein will dictate the particulars of expression, production, and purification methods. For instance, production of proteins for use in screening for modulating agents may require methods that preserve specific biological activities of these proteins, whereas production of proteins for antibody generation may require structural integrity of particular 30 epitopes. Expression of proteins to be purified for screening or antibody production may require the addition of specific tags (*e.g.*, generation of fusion proteins). Overexpression of a MAX protein for assays used to assess MAX function, such as involvement in cell cycle regulation or hypoxic response, may require expression in eukaryotic cell lines capable of these cellular activities. Techniques for the expression, production, and

purification of proteins are well known in the art; any suitable means therefore may be used (e.g., Higgins SJ and Hames BD (eds.) Protein Expression: A Practical Approach, Oxford University Press Inc., New York 1999; Stanbury PF et al., Principles of Fermentation Technology, 2nd edition, Elsevier Science, New York, 1995; Doonan S (ed.)
5 Protein Purification Protocols, Humana Press, New Jersey, 1996; Coligan JE et al, Current Protocols in Protein Science (eds.), 1999, John Wiley & Sons, New York). In particular embodiments, recombinant MAX is expressed in a cell line known to have defective AXIN function. The recombinant cells are used in cell-based screening assay systems of the invention, as described further below.

10 The nucleotide sequence encoding a MAX polypeptide can be inserted into any appropriate expression vector. The necessary transcriptional and translational signals, including promoter/enhancer element, can derive from the native MAX gene and/or its flanking regions or can be heterologous. A variety of host-vector expression systems may be utilized, such as mammalian cell systems infected with virus (e.g. vaccinia virus,
15 adenovirus, *etc.*); insect cell systems infected with virus (e.g. baculovirus); microorganisms such as yeast containing yeast vectors, or bacteria transformed with bacteriophage, plasmid, or cosmid DNA. An isolated host cell strain that modulates the expression of, modifies, and/or specifically processes the gene product may be used.

To detect expression of the MAX gene product, the expression vector can comprise
20 a promoter operably linked to a MAX gene nucleic acid, one or more origins of replication, and, one or more selectable markers (e.g. thymidine kinase activity, resistance to antibiotics, *etc.*). Alternatively, recombinant expression vectors can be identified by assaying for the expression of the MAX gene product based on the physical or functional properties of the MAX protein in *in vitro* assay systems (e.g. immunoassays).

25 The MAX protein, fragment, or derivative may be optionally expressed as a fusion, or chimeric protein product (i.e. it is joined via a peptide bond to a heterologous protein sequence of a different protein), for example to facilitate purification or detection. A chimeric product can be made by ligating the appropriate nucleic acid sequences encoding the desired amino acid sequences to each other using standard methods and expressing the
30 chimeric product. A chimeric product may also be made by protein synthetic techniques, e.g. by use of a peptide synthesizer (Hunkapiller *et al.*, Nature (1984) 310:105-111).

Once a recombinant cell that expresses the MAX gene sequence is identified, the gene product can be isolated and purified using standard methods (e.g. ion exchange, affinity, and gel exclusion chromatography; centrifugation; differential solubility;

electrophoresis). Alternatively, native MAX proteins can be purified from natural sources, by standard methods (*e.g.* immunoaffinity purification). Once a protein is obtained, it may be quantified and its activity measured by appropriate methods, such as immunoassay, bioassay, or other measurements of physical properties, such as crystallography.

5 The methods of this invention may also use cells that have been engineered for altered expression (mis-expression) of MAX or other genes associated with the AXIN pathway. As used herein, mis-expression encompasses ectopic expression, over-expression, under-expression, and non-expression (*e.g.* by gene knock-out or blocking expression that would otherwise normally occur).

10

Genetically modified animals

Animal models that have been genetically modified to alter MAX expression may be used in *in vivo* assays to test for activity of a candidate AXIN modulating agent, or to further assess the role of MAX in a AXIN pathway process such as apoptosis or cell proliferation. Preferably, the altered MAX expression results in a detectable phenotype, such as decreased or increased levels of cell proliferation, angiogenesis, or apoptosis compared to control animals having normal MAX expression. The genetically modified animal may additionally have altered AXIN expression (*e.g.* AXIN knockout). Preferred genetically modified animals are mammals such as primates, rodents (preferably mice or rats), among others. Preferred non-mammalian species include zebrafish, *C. elegans*, and *Drosophila*. Preferred genetically modified animals are transgenic animals having a heterologous nucleic acid sequence present as an extrachromosomal element in a portion of its cells, *i.e.* mosaic animals (see, for example, techniques described by Jakobovits, 1994, Curr. Biol. 4:761-763.) or stably integrated into its germ line DNA (*i.e.*, in the genomic sequence of most or all of its cells). Heterologous nucleic acid is introduced into the germ line of such transgenic animals by genetic manipulation of, for example, embryos or embryonic stem cells of the host animal.

Methods of making transgenic animals are well-known in the art (for transgenic mice see Brinster et al., Proc. Nat. Acad. Sci. USA 82: 4438-4442 (1985), U.S. Pat. Nos. 4,736,866 and 4,870,009, both by Leder et al., U.S. Pat. No. 4,873,191 by Wagner et al., and Hogan, B., Manipulating the Mouse Embryo, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., (1986); for particle bombardment see U.S. Pat. No., 4,945,050, by Sanford *et al.*; for transgenic *Drosophila* see Rubin and Spradling, Science (1982) 218:348-53 and U.S. Pat. No. 4,670,388; for transgenic insects see Berghammer A.J. *et*

al., A Universal Marker for Transgenic Insects (1999) Nature 402:370-371; for transgenic Zebrafish see Lin S., Transgenic Zebrafish, Methods Mol Biol. (2000);136:375-3830; for microinjection procedures for fish, amphibian eggs and birds see Houdebine and Chourrout, Experientia (1991) 47:897-905; for transgenic rats see Hammer *et al.*, Cell (1990) 63:1099-1112; and for culturing of embryonic stem (ES) cells and the subsequent production of transgenic animals by the introduction of DNA into ES cells using methods such as electroporation, calcium phosphate/DNA precipitation and direct injection see, e.g., Teratocarcinomas and Embryonic Stem Cells, A Practical Approach, E. J. Robertson, ed., IRL Press (1987)). Clones of the nonhuman transgenic animals can be produced according to available methods (see Wilmut, I. *et al.* (1997) Nature 385:810-813; and PCT International Publication Nos. WO 97/07668 and WO 97/07669).

In one embodiment, the transgenic animal is a “knock-out” animal having a heterozygous or homozygous alteration in the sequence of an endogenous MAX gene that results in a decrease of MAX function, preferably such that MAX expression is undetectable or insignificant. Knock-out animals are typically generated by homologous recombination with a vector comprising a transgene having at least a portion of the gene to be knocked out. Typically a deletion, addition or substitution has been introduced into the transgene to functionally disrupt it. The transgene can be a human gene (e.g., from a human genomic clone) but more preferably is an ortholog of the human gene derived from the transgenic host species. For example, a mouse MAX gene is used to construct a homologous recombination vector suitable for altering an endogenous MAX gene in the mouse genome. Detailed methodologies for homologous recombination in mice are available (see Capecchi, Science (1989) 244:1288-1292; Joyner *et al.*, Nature (1989) 338:153-156). Procedures for the production of non-rodent transgenic mammals and other animals are also available (Houdebine and Chourrout, *supra*; Pursel *et al.*, Science (1989) 244:1281-1288; Simms *et al.*, Bio/Technology (1988) 6:179-183). In a preferred embodiment, knock-out animals, such as mice harboring a knockout of a specific gene, may be used to produce antibodies against the human counterpart of the gene that has been knocked out (Claesson MH *et al.*, (1994) Scan J Immunol 40:257-264; Declerck PJ *et al.*, (1995) J Biol Chem. 270:8397-400).

In another embodiment, the transgenic animal is a “knock-in” animal having an alteration in its genome that results in altered expression (e.g., increased (including ectopic) or decreased expression) of the MAX gene, e.g., by introduction of additional copies of MAX, or by operatively inserting a regulatory sequence that provides for altered

expression of an endogenous copy of the MAX gene. Such regulatory sequences include inducible, tissue-specific, and constitutive promoters and enhancer elements. The knock-in can be homozygous or heterozygous.

Transgenic nonhuman animals can also be produced that contain selected systems allowing for regulated expression of the transgene. One example of such a system that may be produced is the cre/loxP recombinase system of bacteriophage P1 (Lakso *et al.*, PNAS (1992) 89:6232-6236; U.S. Pat. No. 4,959,317). If a cre/loxP recombinase system is used to regulate expression of the transgene, animals containing transgenes encoding both the Cre recombinase and a selected protein are required. Such animals can be provided through the construction of "double" transgenic animals, e.g., by mating two transgenic animals, one containing a transgene encoding a selected protein and the other containing a transgene encoding a recombinase. Another example of a recombinase system is the FLP recombinase system of *Saccharomyces cerevisiae* (O'Gorman *et al.* (1991) Science 251:1351-1355; U.S. Pat. No. 5,654,182). In a preferred embodiment, both Cre-LoxP and Flp-Frt are used in the same system to regulate expression of the transgene, and for sequential deletion of vector sequences in the same cell (Sun X *et al* (2000) Nat Genet 25:83-6).

The genetically modified animals can be used in genetic studies to further elucidate the AXIN pathway, as animal models of disease and disorders implicating defective AXIN function, and for *in vivo* testing of candidate therapeutic agents, such as those identified in screens described below. The candidate therapeutic agents are administered to a genetically modified animal having altered MAX function and phenotypic changes are compared with appropriate control animals such as genetically modified animals that receive placebo treatment, and/or animals with unaltered MAX expression that receive candidate therapeutic agent.

In addition to the above-described genetically modified animals having altered MAX function, animal models having defective AXIN function (and otherwise normal MAX function), can be used in the methods of the present invention. Preferably, the candidate AXIN modulating agent when administered to a model system with cells defective in AXIN function, produces a detectable phenotypic change in the model system indicating that the AXIN function is restored, i.e., the cells exhibit normal cell cycle progression.

Modulating Agents

The invention provides methods to identify agents that interact with and/or modulate the function of MAX and/or the AXIN pathway. Modulating agents identified by the methods are also part of the invention. Such agents are useful in a variety of diagnostic and therapeutic applications associated with the AXIN pathway, as well as in

further analysis of the MAX protein and its contribution to the AXIN pathway. Accordingly, the invention also provides methods for modulating the AXIN pathway comprising the step of specifically modulating MAX activity by administering a MAX-interacting or -modulating agent.

As used herein, an "MAX-modulating agent" is any agent that modulates MAX function, for example, an agent that interacts with MAX to inhibit or enhance MAX activity or otherwise affect normal MAX function. MAX function can be affected at any level, including transcription, protein expression, protein localization, and cellular or extra-cellular activity. In a preferred embodiment, the MAX - modulating agent specifically modulates the function of the MAX. The phrases "specific modulating agent", "specifically modulates", etc., are used herein to refer to modulating agents that directly bind to the MAX polypeptide or nucleic acid, and preferably inhibit, enhance, or otherwise alter, the function of the MAX. These phrases also encompass modulating agents that alter the interaction of the MAX with a binding partner, substrate, or cofactor (e.g. by binding to a binding partner of a MAX, or to a protein/binding partner complex, and altering MAX function). In a further preferred embodiment, the MAX- modulating agent is a modulator of the AXIN pathway (e.g. it restores and/or upregulates AXIN function) and thus is also an AXIN-modulating agent.

Preferred MAX-modulating agents include small molecule compounds; MAX-interacting proteins, including antibodies and other biotherapeutics; and nucleic acid modulators such as antisense and RNA inhibitors. The modulating agents may be formulated in pharmaceutical compositions, for example, as compositions that may comprise other active ingredients, as in combination therapy, and/or suitable carriers or excipients. Techniques for formulation and administration of the compounds may be found in "Remington's Pharmaceutical Sciences" Mack Publishing Co., Easton, PA, 19th edition.

Small molecule modulators

Small molecules are often preferred to modulate function of proteins with enzymatic function, and/or containing protein interaction domains. Chemical agents, referred to in the art as "small molecule" compounds are typically organic, non-peptide molecules, having a molecular weight less than 10,000, preferably less than 5,000, more preferably less than 1,000, and most preferably less than 500 daltons. This class of modulators includes chemically synthesized molecules, for instance, compounds from combinatorial chemical libraries. Synthetic compounds may be rationally designed or identified based on known or inferred properties of the MAX protein or may be identified by screening compound libraries. Alternative appropriate modulators of this class are natural products, particularly secondary metabolites from organisms such as plants or fungi, which can also be identified by screening compound libraries for MAX-modulating activity. Methods for generating and obtaining compounds are well known in the art (Schreiber SL, Science (2000) 151: 1964-1969; Radmann J and Gunther J, Science (2000) 151:1947-1948).

Small molecule modulators identified from screening assays, as described below, can be used as lead compounds from which candidate clinical compounds may be designed, optimized, and synthesized. Such clinical compounds may have utility in treating pathologies associated with the AXIN pathway. The activity of candidate small molecule modulating agents may be improved several-fold through iterative secondary functional validation, as further described below, structure determination, and candidate modulator modification and testing. Additionally, candidate clinical compounds are generated with specific regard to clinical and pharmacological properties. For example, the reagents may be derivatized and re-screened using *in vitro* and *in vivo* assays to optimize activity and minimize toxicity for pharmaceutical development.

Protein Modulators

Specific MAX-interacting proteins are useful in a variety of diagnostic and therapeutic applications related to the AXIN pathway and related disorders, as well as in validation assays for other MAX-modulating agents. In a preferred embodiment, MAX-interacting proteins affect normal MAX function, including transcription, protein expression, protein localization, and cellular or extra-cellular activity. In another embodiment, MAX-interacting proteins are useful in detecting and providing information

about the function of MAX proteins, as is relevant to AXIN related disorders, such as cancer (e.g., for diagnostic means).

An MAX-interacting protein may be endogenous, i.e. one that naturally interacts genetically or biochemically with a MAX, such as a member of the MAX pathway that modulates MAX expression, localization, and/or activity. MAX-modulators include dominant negative forms of MAX-interacting proteins and of MAX proteins themselves. Yeast two-hybrid and variant screens offer preferred methods for identifying endogenous MAX-interacting proteins (Finley, R. L. et al. (1996) in DNA Cloning-Expression Systems: A Practical Approach, eds. Glover D. & Hames B. D (Oxford University Press, Oxford, England), pp. 169-203; Fashema SF et al., Gene (2000) 250:1-14; Drees BL Curr Opin Chem Biol (1999) 3:64-70; Vidal M and Legrain P Nucleic Acids Res (1999) 27:919-29; and U.S. Pat. No. 5,928,868). Mass spectrometry is an alternative preferred method for the elucidation of protein complexes (reviewed in, e.g., Pandley A and Mann M, Nature (2000) 405:837-846; Yates JR 3rd, Trends Genet (2000) 16:5-8).

An MAX-interacting protein may be an exogenous protein, such as a MAX-specific antibody or a T-cell antigen receptor (see, e.g., Harlow and Lane (1988) Antibodies, A Laboratory Manual, Cold Spring Harbor Laboratory; Harlow and Lane (1999) Using antibodies: a laboratory manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press). MAX antibodies are further discussed below.

In preferred embodiments, a MAX-interacting protein specifically binds a MAX protein. In alternative preferred embodiments, a MAX-modulating agent binds a MAX substrate, binding partner, or cofactor.

Antibodies

In another embodiment, the protein modulator is a MAX specific antibody agonist or antagonist. The antibodies have therapeutic and diagnostic utilities, and can be used in screening assays to identify MAX modulators. The antibodies can also be used in dissecting the portions of the MAX pathway responsible for various cellular responses and in the general processing and maturation of the MAX.

Antibodies that specifically bind MAX polypeptides can be generated using known methods. Preferably the antibody is specific to a mammalian ortholog of MAX polypeptide, and more preferably, to human MAX. Antibodies may be polyclonal, monoclonal (mAbs), humanized or chimeric antibodies, single chain antibodies, Fab fragments, F(ab').sub.2 fragments, fragments produced by a FAb expression library, anti-

idiotypic (anti-Id) antibodies, and epitope-binding fragments of any of the above.

Epitopes of MAX which are particularly antigenic can be selected, for example, by routine screening of MAX polypeptides for antigenicity or by applying a theoretical method for selecting antigenic regions of a protein (Hopp and Wood (1981), Proc. Natl. Acad. Sci. U.S.A. 78:3824-28; Hopp and Wood, (1983) Mol. Immunol. 20:483-89; Sutcliffe et al., (1983) Science 219:660-66) to the amino acid sequence of a MAX. Monoclonal antibodies with affinities of 10^8 M^{-1} preferably 10^9 M^{-1} to 10^{10} M^{-1} , or stronger can be made by standard procedures as described (Harlow and Lane, *supra*; Goding (1986) Monoclonal Antibodies: Principles and Practice (2d ed) Academic Press, New York; and U.S. Pat. Nos. 4,381,292; 4,451,570; and 4,618,577). Antibodies may be generated against crude cell extracts of MAX or substantially purified fragments thereof. If MAX fragments are used, they preferably comprise at least 10, and more preferably, at least 20 contiguous amino acids of a MAX protein. In a particular embodiment, MAX-specific antigens and/or immunogens are coupled to carrier proteins that stimulate the immune response. For example, the subject polypeptides are covalently coupled to the keyhole limpet hemocyanin (KLH) carrier, and the conjugate is emulsified in Freund's complete adjuvant, which enhances the immune response. An appropriate immune system such as a laboratory rabbit or mouse is immunized according to conventional protocols.

The presence of MAX-specific antibodies is assayed by an appropriate assay such as a solid phase enzyme-linked immunosorbant assay (ELISA) using immobilized corresponding MAX polypeptides. Other assays, such as radioimmunoassays or fluorescent assays might also be used.

Chimeric antibodies specific to MAX polypeptides can be made that contain different portions from different animal species. For instance, a human immunoglobulin constant region may be linked to a variable region of a murine mAb, such that the antibody derives its biological activity from the human antibody, and its binding specificity from the murine fragment. Chimeric antibodies are produced by splicing together genes that encode the appropriate regions from each species (Morrison et al., Proc. Natl. Acad. Sci. (1984) 81:6851-6855; Neuberger et al., Nature (1984) 312:604-608; Takeda et al., Nature (1985) 31:452-454). Humanized antibodies, which are a form of chimeric antibodies, can be generated by grafting complementary-determining regions (CDRs) (Carlos, T. M., J. M. Harlan. 1994. Blood 84:2068-2101) of mouse antibodies into a background of human framework regions and constant regions by recombinant DNA technology (Riechmann LM, et al., 1988 Nature 323: 323-327). Humanized

antibodies contain ~10% murine sequences and ~90% human sequences, and thus further reduce or eliminate immunogenicity, while retaining the antibody specificities (Co MS, and Queen C. 1991 Nature 351: 501-501; Morrison SL. 1992 Ann. Rev. Immun. 10:239-265). Humanized antibodies and methods of their production are well-known in the art (U.S. Pat. Nos. 5,530,101, 5,585,089, 5,693,762, and 6,180,370).

MAX-specific single chain antibodies which are recombinant, single chain polypeptides formed by linking the heavy and light chain fragments of the Fv regions via an amino acid bridge, can be produced by methods known in the art (U.S. Pat. No. 4,946,778; Bird, Science (1988) 242:423-426; Huston et al., Proc. Natl. Acad. Sci. USA (1988) 85:5879-5883; and Ward et al., Nature (1989) 334:544-546).

Other suitable techniques for antibody production involve in vitro exposure of lymphocytes to the antigenic polypeptides or alternatively to selection of libraries of antibodies in phage or similar vectors (Huse et al., Science (1989) 246:1275-1281). As used herein, T-cell antigen receptors are included within the scope of antibody modulators (Harlow and Lane, 1988, *supra*).

The polypeptides and antibodies of the present invention may be used with or without modification. Frequently, antibodies will be labeled by joining, either covalently or non-covalently, a substance that provides for a detectable signal, or that is toxic to cells that express the targeted protein (Menard S, et al., Int J. Biol Markers (1989) 4:131-134). A wide variety of labels and conjugation techniques are known and are reported extensively in both the scientific and patent literature. Suitable labels include radionuclides, enzymes, substrates, cofactors, inhibitors, fluorescent moieties, fluorescent emitting lanthanide metals, chemiluminescent moieties, bioluminescent moieties, magnetic particles, and the like (U.S. Pat. Nos. 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149; and 4,366,241). Also, recombinant immunoglobulins may be produced (U.S. Pat. No. 4,816,567). Antibodies to cytoplasmic polypeptides may be delivered and reach their targets by conjugation with membrane-penetrating toxin proteins (U.S. Pat. No. 6,086,900).

When used therapeutically in a patient, the antibodies of the subject invention are typically administered parenterally, when possible at the target site, or intravenously. The therapeutically effective dose and dosage regimen is determined by clinical studies. Typically, the amount of antibody administered is in the range of about 0.1 mg/kg –to about 10 mg/kg of patient weight. For parenteral administration, the antibodies are formulated in a unit dosage injectable form (e.g., solution, suspension, emulsion) in

association with a pharmaceutically acceptable vehicle. Such vehicles are inherently nontoxic and non-therapeutic. Examples are water, saline, Ringer's solution, dextrose solution, and 5% human serum albumin. Nonaqueous vehicles such as fixed oils, ethyl oleate, or liposome carriers may also be used. The vehicle may contain minor amounts of additives, such as buffers and preservatives, which enhance isotonicity and chemical stability or otherwise enhance therapeutic potential. The antibodies' concentrations in such vehicles are typically in the range of about 1 mg/ml to about 10 mg/ml. Immunotherapeutic methods are further described in the literature (US Pat. No. 5,859,206; WO0073469).

Specific biotherapeutics

In a preferred embodiment, a MAX-interacting protein may have biotherapeutic applications. Biotherapeutic agents formulated in pharmaceutically acceptable carriers and dosages may be used to activate or inhibit signal transduction pathways. This modulation may be accomplished by binding a ligand, thus inhibiting the activity of the pathway; or by binding a receptor, either to inhibit activation of, or to activate, the receptor. Alternatively, the biotherapeutic may itself be a ligand capable of activating or inhibiting a receptor. Biotherapeutic agents and methods of producing them are described in detail in U.S. Pat. No. 6,146,628.

When the MAX is a ligand, it may be used as a biotherapeutic agent to activate or inhibit its natural receptor. Alternatively, antibodies against MAX, as described in the previous section, may be used as biotherapeutic agents.

When the MAX is a receptor, its ligand(s), antibodies to the ligand(s) or the MAX itself may be used as biotherapeutics to modulate the activity of MAX in the AXIN pathway.

Nucleic Acid Modulators

Other preferred MAX-modulating agents comprise nucleic acid molecules, such as antisense oligomers or double stranded RNA (dsRNA), which generally inhibit MAX activity. Preferred nucleic acid modulators interfere with the function of the MAX nucleic acid such as DNA replication, transcription, translocation of the MAX RNA to the site of protein translation, translation of protein from the MAX RNA, splicing of the MAX RNA to yield one or more mRNA species, or catalytic activity which may be engaged in or facilitated by the MAX RNA.

In one embodiment, the antisense oligomer is an oligonucleotide that is sufficiently complementary to a MAX mRNA to bind to and prevent translation, preferably by binding to the 5' untranslated region. MAX-specific antisense oligonucleotides, preferably range from at least 6 to about 200 nucleotides. In some embodiments the oligonucleotide is preferably at least 10, 15, or 20 nucleotides in length. In other embodiments, the oligonucleotide is preferably less than 50, 40, or 30 nucleotides in length. The oligonucleotide can be DNA or RNA or a chimeric mixture or derivatives or modified versions thereof, single-stranded or double-stranded. The oligonucleotide can be modified at the base moiety, sugar moiety, or phosphate backbone. The oligonucleotide may include other appending groups such as peptides, agents that facilitate transport across the cell membrane, hybridization-triggered cleavage agents, and intercalating agents.

In another embodiment, the antisense oligomer is a phosphothioate morpholino oligomer (PMO). PMOs are assembled from four different morpholino subunits, each of which contain one of four genetic bases (A, C, G, or T) linked to a six-membered morpholine ring. Polymers of these subunits are joined by non-ionic phosphodiarnidate intersubunit linkages. Details of how to make and use PMOs and other antisense oligomers are well known in the art (e.g. see WO99/18193; Probst JC, Antisense Oligodeoxynucleotide and Ribozyme Design, Methods. (2000) 22(3):271-281; Summerton J, and Weller D. 1997 Antisense Nucleic Acid Drug Dev. :7:187-95; US Pat. No. 5,235,033; and US Pat No. 5,378,841).

Alternative preferred MAX nucleic acid modulators are double-stranded RNA species mediating RNA interference (RNAi). RNAi is the process of sequence-specific, post-transcriptional gene silencing in animals and plants, initiated by double-stranded RNA (dsRNA) that is homologous in sequence to the silenced gene. Methods relating to the use of RNAi to silence genes in *C. elegans*, *Drosophila*, plants, and humans are known in the art (Fire A, et al., 1998 Nature 391:806-811; Fire, A. Trends Genet. 15, 358-363 (1999); Sharp, P. A. RNA interference 2001. Genes Dev. 15, 485-490 (2001); Hammond, S. M., et al., Nature Rev. Genet. 2, 110-1119 (2001); Tuschl, T. Chem. Biochem. 2, 239-245 (2001); Hamilton, A. et al., Science 286, 950-952 (1999); Hammond, S. M., et al., Nature 404, 293-296 (2000); Zamore, P. D., et al., Cell 101, 25-33 (2000); Bernstein, E., et al., Nature 409, 363-366 (2001); Elbashir, S. M., et al., Genes Dev. 15, 188-200 (2001); WO0129058; WO9932619; Elbashir SM, et al., 2001 Nature 411:494-498).

Nucleic acid modulators are commonly used as research reagents, diagnostics, and therapeutics. For example, antisense oligonucleotides, which are able to inhibit gene

expression with exquisite specificity, are often used to elucidate the function of particular genes (see, for example, U.S. Pat. No. 6,165,790). Nucleic acid modulators are also used, for example, to distinguish between functions of various members of a biological pathway. For example, antisense oligomers have been employed as therapeutic moieties in the treatment of disease states in animals and man and have been demonstrated in numerous clinical trials to be safe and effective (Milligan JF, *et al*, Current Concepts in Antisense Drug Design, J Med Chem. (1993) 36:1923-1937; Tonkinson JL *et al.*, Antisense Oligodeoxynucleotides as Clinical Therapeutic Agents, Cancer Invest. (1996) 14:54-65). Accordingly, in one aspect of the invention, a MAX-specific nucleic acid modulator is used in an assay to further elucidate the role of the MAX in the AXIN pathway, and/or its relationship to other members of the pathway. In another aspect of the invention, a MAX-specific antisense oligomer is used as a therapeutic agent for treatment of AXIN-related disease states.

15 Assay Systems

The invention provides assay systems and screening methods for identifying specific modulators of MAX activity. As used herein, an "assay system" encompasses all the components required for performing and analyzing results of an assay that detects and/or measures a particular event. In general, primary assays are used to identify or confirm a modulator's specific biochemical or molecular effect with respect to the MAX nucleic acid or protein. In general, secondary assays further assess the activity of a MAX modulating agent identified by a primary assay and may confirm that the modulating agent affects MAX in a manner relevant to the AXIN pathway. In some cases, MAX modulators will be directly tested in a secondary assay.

25 In a preferred embodiment, the screening method comprises contacting a suitable assay system comprising a MAX polypeptide or nucleic acid with a candidate agent under conditions whereby, but for the presence of the agent, the system provides a reference activity (e.g. kbinding activity), which is based on the particular molecular event the screening method detects. A statistically significant difference between the agent-biased activity and the reference activity indicates that the candidate agent modulates MAX activity, and hence the AXIN pathway. The MAX polypeptide or nucleic acid used in the assay may comprise any of the nucleic acids or polypeptides described above.

Primary Assays

The type of modulator tested generally determines the type of primary assay.

Primary assays for small molecule modulators

5 For small molecule modulators, screening assays are used to identify candidate modulators. Screening assays may be cell-based or may use a cell-free system that recreates or retains the relevant biochemical reaction of the target protein (reviewed in Sittampalam GS *et al.*, Curr Opin Chem Biol (1997) 1:384-91 and accompanying references). As used herein the term "cell-based" refers to assays using live cells, dead
10 cells, or a particular cellular fraction, such as a membrane, endoplasmic reticulum, or mitochondrial fraction. The term "cell free" encompasses assays using substantially purified protein (either endogenous or recombinantly produced), partially purified or crude cellular extracts. Screening assays may detect a variety of molecular events, including protein-DNA interactions, protein-protein interactions (*e.g.*, receptor-ligand binding),
15 transcriptional activity (*e.g.*, using a reporter gene), enzymatic activity (*e.g.*, via a property of the substrate), activity of second messengers, immunogenicity and changes in cellular morphology or other cellular characteristics. Appropriate screening assays may use a wide range of detection methods including fluorescent, radioactive, colorimetric, spectrophotometric, and amperometric methods, to provide a read-out for the particular
20 molecular event detected.

Cell-based screening assays usually require systems for recombinant expression of MAX and any auxiliary proteins demanded by the particular assay. Appropriate methods for generating recombinant proteins produce sufficient quantities of proteins that retain their relevant biological activities and are of sufficient purity to optimize activity and
25 assure assay reproducibility. Yeast two-hybrid and variant screens, and mass spectrometry provide preferred methods for determining protein-protein interactions and elucidation of protein complexes. In certain applications, when MAX-interacting proteins are used in screens to identify small molecule modulators, the binding specificity of the interacting protein to the MAX protein may be assayed by various known methods such as substrate
30 processing (*e.g.* ability of the candidate MAX-specific binding agents to function as negative effectors in MAX-expressing cells), binding equilibrium constants (usually at least about 10^7 M^{-1} , preferably at least about 10^8 M^{-1} , more preferably at least about 10^9 M^{-1}), and immunogenicity (*e.g.* ability to elicit MAX specific antibody in a heterologous host

such as a mouse, rat, goat or rabbit). For enzymes and receptors, binding may be assayed by, respectively, substrate and ligand processing.

The screening assay may measure a candidate agent's ability to specifically bind to or modulate activity of a MAX polypeptide, a fusion protein thereof, or to cells or
5 membranes bearing the polypeptide or fusion protein. The MAX polypeptide can be full length or a fragment thereof that retains functional MAX activity. The MAX polypeptide may be fused to another polypeptide, such as a peptide tag for detection or anchoring, or to another tag. The MAX polypeptide is preferably human MAX, or is an ortholog or derivative thereof as described above. In a preferred embodiment, the screening assay
10 detects candidate agent-based modulation of MAX interaction with a binding target, such as an endogenous or exogenous protein or other substrate that has MAX -specific binding activity, and can be used to assess normal MAX gene function.

Suitable assay formats that may be adapted to screen for MAX modulators are known in the art. Preferred screening assays are high throughput or ultra high throughput
15 and thus provide automated, cost-effective means of screening compound libraries for lead compounds (Fernandes PB, Curr Opin Chem Biol (1998) 2:597-603; Sundberg SA, Curr Opin Biotechnol 2000, 11:47-53). In one preferred embodiment, screening assays uses fluorescence technologies, including fluorescence polarization, time-resolved fluorescence, and fluorescence resonance energy transfer. These systems offer means to
20 monitor protein-protein or DNA-protein interactions in which the intensity of the signal emitted from dye-labeled molecules depends upon their interactions with partner molecules (*e.g.*, Selvin PR, Nat Struct Biol (2000) 7:730-4; Fernandes PB, *supra*; Hertzberg RP and Pope AJ, Curr Opin Chem Biol (2000) 4:445-451).

A variety of suitable assay systems may be used to identify candidate MAX and
25 AXIN pathway modulators (*e.g.* U.S. Pat. No. 6,165,992 (kinase assays); U.S. Pat. Nos. 5,550,019 and 6,133,437 (apoptosis assays); U.S. Pat. No. 6,114,132 (phosphatase and protease assays), U.S. Pat. Nos. 5,976,782, 6,225,118 and 6,444,434 (angiogenesis assays), among others). Specific preferred assays are described in more detail below.

Proteases are enzymes that cleave protein substrates at specific sites. Exemplary
30 assays detect the alterations in the spectral properties of an artificial substrate that occur upon protease-mediated cleavage. In one example, synthetic caspase substrates containing four amino acid proteolysis recognition sequences, separating two different fluorescent tags are employed; fluorescence resonance energy transfer detects the proximity of these

fluorophores, which indicates whether the substrate is cleaved (Mahajan NP *et al.*, Chem Biol (1999) 6:401-409).

Kinase assays. In some preferred embodiments the screening assay detects the ability of the test agent to modulate the kinase activity of a MAX polypeptide. In further embodiments, a cell-free kinase assay system is used to identify a candidate AXIN modulating agent, and a secondary, cell-based assay, such as an apoptosis or hypoxic induction assay (described below), may be used to further characterize the candidate AXIN modulating agent. Many different assays for kinases have been reported in the literature and are well known to those skilled in the art (e.g. U.S. Pat. No. 6,165,992; Zhu et al., Nature Genetics (2000) 26:283-289; and WO0073469). Radioassays, which monitor the transfer of a gamma phosphate are frequently used. For instance, a scintillation assay for p56 (lck) kinase activity monitors the transfer of the gamma phosphate from gamma -³³P ATP to a biotinylated peptide substrate; the substrate is captured on a streptavidin coated bead that transmits the signal (Beveridge M *et al.*, J Biomol Screen (2000) 5:205-212). This assay uses the scintillation proximity assay (SPA), in which only radio-ligand bound to receptors tethered to the surface of an SPA bead are detected by the scintillant immobilized within it, allowing binding to be measured without separation of bound from free ligand.

Other assays for protein kinase activity may use antibodies that specifically recognize phosphorylated substrates. For instance, the kinase receptor activation (KIRA) assay measures receptor tyrosine kinase activity by ligand stimulating the intact receptor in cultured cells, then capturing solubilized receptor with specific antibodies and quantifying phosphorylation via phosphotyrosine ELISA (Sadick MD, Dev Biol Stand (1999) 97:121-133).

Another example of antibody based assays for protein kinase activity is TRF (time-resolved fluorometry). This method utilizes europium chelate-labeled anti-phosphotyrosine antibodies to detect phosphate transfer to a polymeric substrate coated onto microtiter plate wells. The amount of phosphorylation is then detected using time-resolved, dissociation-enhanced fluorescence (Braunwalder AF, et al., Anal Biochem 1996 Jul 1;238(2):159-64).

Apoptosis assays. Assays for apoptosis may be performed by terminal deoxynucleotidyl transferase-mediated digoxigenin-11-dUTP nick end labeling (TUNEL)

assay. The TUNEL assay is used to measure nuclear DNA fragmentation characteristic of apoptosis (Lazebnik *et al.*, 1994, Nature 371, 346), by following the incorporation of fluorescein-dUTP (Yonehara *et al.*, 1989, J. Exp. Med. 169, 1747). Apoptosis may further be assayed by acridine orange staining of tissue culture cells (Lucas, R., et al., 1998, Blood 5 15:4730-41). Other cell-based apoptosis assays include the caspase-3/7 assay and the cell death nucleosome ELISA assay. The caspase 3/7 assay is based on the activation of the caspase cleavage activity as part of a cascade of events that occur during programmed cell death in many apoptotic pathways. In the caspase 3/7 assay (commercially available Apo-ONE™ Homogeneous Caspase-3/7 assay from Promega, cat# 67790), lysis buffer and 10 caspase substrate are mixed and added to cells. The caspase substrate becomes fluorescent when cleaved by active caspase 3/7. The nucleosome ELISA assay is a general cell death assay known to those skilled in the art, and available commercially (Roche, Cat# 1774425). This assay is a quantitative sandwich-enzyme-immunoassay which uses monoclonal antibodies directed against DNA and histones respectively, thus specifically 15 determining amount of mono- and oligonucleosomes in the cytoplasmic fraction of cell lysates. Mono and oligonucleosomes are enriched in the cytoplasm during apoptosis due to the fact that DNA fragmentation occurs several hours before the plasma membrane breaks down, allowing for accumulation in the cytoplasm. Nucleosomes are not present in the cytoplasmic fraction of cells that are not undergoing apoptosis. An apoptosis assay 20 system may comprise a cell that expresses a MAX, and that optionally has defective AXIN function (e.g. AXIN is over-expressed or under-expressed relative to wild-type cells). A test agent can be added to the apoptosis assay system and changes in induction of apoptosis relative to controls where no test agent is added, identify candidate AXIN modulating agents. In some embodiments of the invention, an apoptosis assay may be 25 used as a secondary assay to test a candidate AXIN modulating agents that is initially identified using a cell-free assay system. An apoptosis assay may also be used to test whether MAX function plays a direct role in apoptosis. For example, an apoptosis assay may be performed on cells that over- or under-express MAX relative to wild type cells. Differences in apoptotic response compared to wild type cells suggests that the MAX 30 plays a direct role in the apoptotic response. Apoptosis assays are described further in US Pat. No. 6,133,437.

Cell proliferation and cell cycle assays. Cell proliferation may be assayed via bromodeoxyuridine (BRDU) incorporation. This assay identifies a cell population

undergoing DNA synthesis by incorporation of BRDU into newly-synthesized DNA. Newly-synthesized DNA may then be detected using an anti-BRDU antibody (Hoshino *et al.*, 1986, Int. J. Cancer 38, 369; Campana *et al.*, 1988, J. Immunol. Meth. 107, 79), or by other means.

5 Cell proliferation is also assayed via phospho-histone H3 staining, which identifies a cell population undergoing mitosis by phosphorylation of histone H3. Phosphorylation of histone H3 at serine 10 is detected using an antibody specific to the phosphorylated form of the serine 10 residue of histone H3. (Chadlee, D.N. 1995, J. Biol. Chem 270:20098-105). Cell Proliferation may also be examined using [³H]-thymidine incorporation (Chen, 10 J., 1996, Oncogene 13:1395-403; Jeoung, J., 1995, J. Biol. Chem. 270:18367-73). This assay allows for quantitative characterization of S-phase DNA syntheses. In this assay, cells synthesizing DNA will incorporate [³H]-thymidine into newly synthesized DNA. Incorporation can then be measured by standard techniques such as by counting of radioisotope in a scintillation counter (e.g., Beckman LS 3800 Liquid Scintillation 15 Counter). Another proliferation assay uses the dye Alamar Blue (available from Biosource International), which fluoresces when reduced in living cells and provides an indirect measurement of cell number (Voytik-Harbin SL *et al.*, 1998, In Vitro Cell Dev Biol Anim 34:239-46). Yet another proliferation assay, the MTS assay, is based on in vitro cytotoxicity assessment of industrial chemicals, and uses the soluble tetrazolium salt, 20 MTS. MTS assays are commercially available, for example, the Promega CellTiter 96[®] AQueous Non-Radioactive Cell Proliferation Assay (Cat.# G5421).

Cell proliferation may also be assayed by colony formation in soft agar (Sambrook *et al.*, Molecular Cloning, Cold Spring Harbor (1989)). For example, cells transformed with MAX are seeded in soft agar plates, and colonies are measured and counted after two 25 weeks incubation.

Cell proliferation may also be assayed by measuring ATP levels as indicator of metabolically active cells. Such assays are commercially available, for example Cell Titer-Glo[™], which is a luminescent homogeneous assay available from Promega.

Involvement of a gene in the cell cycle may be assayed by flow cytometry (Gray 30 JW *et al.* (1986) Int J Radiat Biol Relat Stud Phys Chem Med 49:237-55). Cells transfected with a MAX may be stained with propidium iodide and evaluated in a flow cytometer (available from Becton Dickinson), which indicates accumulation of cells in different stages of the cell cycle.

Accordingly, a cell proliferation or cell cycle assay system may comprise a cell that expresses a MAX, and that optionally has defective AXIN function (e.g. AXIN is over-expressed or under-expressed relative to wild-type cells). A test agent can be added to the assay system and changes in cell proliferation or cell cycle relative to controls where
5 no test agent is added, identify candidate AXIN modulating agents. In some embodiments of the invention, the cell proliferation or cell cycle assay may be used as a secondary assay to test a candidate AXIN modulating agents that is initially identified using another assay system such as a cell-free assay system. A cell proliferation assay may also be used to test whether MAX function plays a direct role in cell proliferation or cell cycle. For example,
10 a cell proliferation or cell cycle assay may be performed on cells that over- or under-express MAX relative to wild type cells. Differences in proliferation or cell cycle compared to wild type cells suggests that the MAX plays a direct role in cell proliferation or cell cycle.

15 **Angiogenesis.** Angiogenesis may be assayed using various human endothelial cell systems, such as umbilical vein, coronary artery, or dermal cells. Suitable assays include Alamar Blue based assays (available from Biosource International) to measure proliferation; migration assays using fluorescent molecules, such as the use of Becton Dickinson Falcon HTS FluoroBlock cell culture inserts to measure migration of cells
20 through membranes in presence or absence of angiogenesis enhancer or suppressors; and tubule formation assays based on the formation of tubular structures by endothelial cells on Matrigel® (Becton Dickinson). Accordingly, an angiogenesis assay system may comprise a cell that expresses a MAX, and that optionally has defective AXIN function (e.g. AXIN is over-expressed or under-expressed relative to wild-type cells). A test agent
25 can be added to the angiogenesis assay system and changes in angiogenesis relative to controls where no test agent is added, identify candidate AXIN modulating agents. In some embodiments of the invention, the angiogenesis assay may be used as a secondary assay to test a candidate AXIN modulating agents that is initially identified using another assay system. An angiogenesis assay may also be used to test whether MAX function
30 plays a direct role in cell proliferation. For example, an angiogenesis assay may be performed on cells that over- or under-express MAX relative to wild type cells. Differences in angiogenesis compared to wild type cells suggests that the MAX plays a direct role in angiogenesis. U.S. Pat. Nos. 5,976,782, 6,225,118 and 6,444,434, among others, describe various angiogenesis assays.

Hypoxic induction. The alpha subunit of the transcription factor, hypoxia inducible factor-1 (HIF-1), is upregulated in tumor cells following exposure to hypoxia in vitro. Under hypoxic conditions, HIF-1 stimulates the expression of genes known to be important in tumour cell survival, such as those encoding glycolytic enzymes and VEGF.

5 Induction of such genes by hypoxic conditions may be assayed by growing cells transfected with MAX in hypoxic conditions (such as with 0.1% O₂, 5% CO₂, and balance N₂, generated in a Napco 7001 incubator (Precision Scientific)) and normoxic conditions, followed by assessment of gene activity or expression by Taqman®. For example, a hypoxic induction assay system may comprise a cell that expresses a MAX,

10 and that optionally has defective AXIN function (e.g. AXIN is over-expressed or under-expressed relative to wild-type cells). A test agent can be added to the hypoxic induction assay system and changes in hypoxic response relative to controls where no test agent is added, identify candidate AXIN modulating agents. In some embodiments of the invention, the hypoxic induction assay may be used as a secondary assay to test a

15 candidate AXIN modulating agents that is initially identified using another assay system. A hypoxic induction assay may also be used to test whether MAX function plays a direct role in the hypoxic response. For example, a hypoxic induction assay may be performed on cells that over- or under-express MAX relative to wild type cells. Differences in hypoxic response compared to wild type cells suggests that the MAX plays a direct role in

20 hypoxic induction.

Cell adhesion. Cell adhesion assays measure adhesion of cells to purified adhesion proteins, or adhesion of cells to each other, in presence or absence of candidate modulating agents. Cell-protein adhesion assays measure the ability of agents to modulate

25 the adhesion of cells to purified proteins. For example, recombinant proteins are produced, diluted to 2.5g/mL in PBS, and used to coat the wells of a microtiter plate. The wells used for negative control are not coated. Coated wells are then washed, blocked with 1% BSA, and washed again. Compounds are diluted to 2× final test concentration and added to the blocked, coated wells. Cells are then added to the wells, and the unbound

30 cells are washed off. Retained cells are labeled directly on the plate by adding a membrane-permeable fluorescent dye, such as calcein-AM, and the signal is quantified in a fluorescent microplate reader.

Cell-cell adhesion assays measure the ability of agents to modulate binding of cell adhesion proteins with their native ligands. These assays use cells that naturally or

recombinantly express the adhesion protein of choice. In an exemplary assay, cells expressing the cell adhesion protein are plated in wells of a multiwell plate. Cells expressing the ligand are labeled with a membrane-permeable fluorescent dye, such as BCECF, and allowed to adhere to the monolayers in the presence of candidate agents.

5 Unbound cells are washed off, and bound cells are detected using a fluorescence plate reader.

High-throughput cell adhesion assays have also been described. In one such assay, small molecule ligands and peptides are bound to the surface of microscope slides using a microarray spotter, intact cells are then contacted with the slides, and unbound cells are
10 washed off. In this assay, not only the binding specificity of the peptides and modulators against cell lines are determined, but also the functional cell signaling of attached cells using immunofluorescence techniques in situ on the microchip is measured (Falsey JR et al., Bioconjug Chem. 2001 May-Jun;12(3):346-53).

15 **Tubulogenesis.** Tubulogenesis assays monitor the ability of cultured cells, generally endothelial cells, to form tubular structures on a matrix substrate, which generally simulates the environment of the extracellular matrix. Exemplary substrates include MatrigelTM (Becton Dickinson), an extract of basement membrane proteins containing laminin, collagen IV, and heparin sulfate proteoglycan, which is liquid at 4° C
20 and forms a solid gel at 37° C. Other suitable matrices comprise extracellular components such as collagen, fibronectin, and/or fibrin. Cells are stimulated with a pro-angiogenic stimulant, and their ability to form tubules is detected by imaging. Tubules can generally be detected after an overnight incubation with stimuli, but longer or shorter time frames may also be used. Tube formation assays are well known in the art (e.g., Jones MK et al.,
25 1999, Nature Medicine 5:1418-1423). These assays have traditionally involved stimulation with serum or with the growth factors FGF or VEGF. Serum represents an undefined source of growth factors. In a preferred embodiment, the assay is performed with cells cultured in serum free medium, in order to control which process or pathway a candidate agent modulates. Moreover, we have found that different target genes respond
30 differently to stimulation with different pro-angiogenic agents, including inflammatory angiogenic factors such as TNF- α . Thus, in a further preferred embodiment, a tubulogenesis assay system comprises testing a MAX's response to a variety of factors, such as FGF, VEGF, phorbol myristate acetate (PMA), TNF- α , ephrin, etc.

Cell Migration. An invasion/migration assay (also called a migration assay) tests the ability of cells to overcome a physical barrier and to migrate towards pro-angiogenic signals. Migration assays are known in the art (e.g., Paik JH et al., 2001, J Biol Chem 276:11830-11837). In a typical experimental set-up, cultured endothelial cells are seeded
5 onto a matrix-coated porous lamina, with pore sizes generally smaller than typical cell size. The matrix generally simulates the environment of the extracellular matrix, as described above. The lamina is typically a membrane, such as the transwell polycarbonate membrane (Corning Costar Corporation, Cambridge, MA), and is generally part of an upper chamber that is in fluid contact with a lower chamber containing pro-angiogenic
10 stimuli. Migration is generally assayed after an overnight incubation with stimuli, but longer or shorter time frames may also be used. Migration is assessed as the number of cells that crossed the lamina, and may be detected by staining cells with hemotoxylin solution (VWR Scientific, South San Francisco, CA), or by any other method for determining cell number. In another exemplary set up, cells are fluorescently labeled and
15 migration is detected using fluorescent readings, for instance using the Falcon HTS FluoroBlok (Becton Dickinson). While some migration is observed in the absence of stimulus, migration is greatly increased in response to pro-angiogenic factors. As described above, a preferred assay system for migration/invasion assays comprises testing a MAX's response to a variety of pro-angiogenic factors, including tumor angiogenic and
20 inflammatory angiogenic agents, and culturing the cells in serum free medium.

Sprouting assay. A sprouting assay is a three-dimensional *in vitro* angiogenesis assay that uses a cell-number defined spheroid aggregation of endothelial cells ("spheroid"), embedded in a collagen gel-based matrix. The spheroid can serve as a
25 starting point for the sprouting of capillary-like structures by invasion into the extracellular matrix (termed "cell sprouting") and the subsequent formation of complex anastomosing networks (Korff and Augustin, 1999, J Cell Sci 112:3249-58). In an exemplary experimental set-up, spheroids are prepared by pipetting 400 human umbilical vein endothelial cells into individual wells of a nonadhesive 96-well plates to allow
30 overnight spheroidal aggregation (Korff and Augustin: J Cell Biol 143: 1341-52, 1998). Spheroids are harvested and seeded in 900 μ l of methocel-collagen solution and pipetted into individual wells of a 24 well plate to allow collagen gel polymerization. Test agents are added after 30 min by pipetting 100 μ l of 10-fold concentrated working dilution of the test substances on top of the gel. Plates are incubated at 37°C for 24h. Dishes are fixed at

the end of the experimental incubation period by addition of paraformaldehyde. Sprouting intensity of endothelial cells can be quantitated by an automated image analysis system to determine the cumulative sprout length per spheroid.

5 ***Primary assays for antibody modulators***

For antibody modulators, appropriate primary assays test is a binding assay that tests the antibody's affinity to and specificity for the MAX protein. Methods for testing antibody affinity and specificity are well known in the art (Harlow and Lane, 1988, 1999, *supra*). The enzyme-linked immunosorbant assay (ELISA) is a preferred method for
10 detecting MAX-specific antibodies; others include FACS assays, radioimmunoassays, and fluorescent assays.

In some cases, screening assays described for small molecule modulators may also be used to test antibody modulators.

15 ***Primary assays for nucleic acid modulators***

For nucleic acid modulators, primary assays may test the ability of the nucleic acid modulator to inhibit or enhance MAX gene expression, preferably mRNA expression. In general, expression analysis comprises comparing MAX expression in like populations of cells (*e.g.*, two pools of cells that endogenously or recombinantly express MAX) in the
20 presence and absence of the nucleic acid modulator. Methods for analyzing mRNA and protein expression are well known in the art. For instance, Northern blotting, slot blotting, ribonuclease protection, quantitative RT-PCR (*e.g.*, using the TaqMan®, PE Applied Biosystems), or microarray analysis may be used to confirm that MAX mRNA expression is reduced in cells treated with the nucleic acid modulator (*e.g.*, Current Protocols in
25 Molecular Biology (1994) Ausubel FM *et al.*, eds., John Wiley & Sons, Inc., chapter 4; Freeman WM *et al.*, Biotechniques (1999) 26:112-125; Kallioniemi OP, Ann Med 2001, 33:142-147; Blohm DH and Guiseppi-Elie, A Curr Opin Biotechnol 2001, 12:41-47). Protein expression may also be monitored. Proteins are most commonly detected with specific antibodies or antisera directed against either the MAX protein or specific peptides.
30 A variety of means including Western blotting, ELISA, or in situ detection, are available (Harlow E and Lane D, 1988 and 1999, *supra*).

In some cases, screening assays described for small molecule modulators, particularly in assay systems that involve MAX mRNA expression, may also be used to test nucleic acid modulators.

Secondary Assays

Secondary assays may be used to further assess the activity of MAX-modulating agent identified by any of the above methods to confirm that the modulating agent affects MAX in a manner relevant to the AXIN pathway. As used herein, MAX-modulating agents encompass candidate clinical compounds or other agents derived from previously identified modulating agent. Secondary assays can also be used to test the activity of a modulating agent on a particular genetic or biochemical pathway or to test the specificity of the modulating agent's interaction with MAX.

Secondary assays generally compare like populations of cells or animals (*e.g.*, two pools of cells or animals that endogenously or recombinantly express MAX) in the presence and absence of the candidate modulator. In general, such assays test whether treatment of cells or animals with a candidate MAX-modulating agent results in changes in the AXIN pathway in comparison to untreated (or mock- or placebo-treated) cells or animals. Certain assays use "sensitized genetic backgrounds", which, as used herein, describe cells or animals engineered for altered expression of genes in the AXIN or interacting pathways.

Cell-based assays

Cell based assays may detect endogenous AXIN pathway activity or may rely on recombinant expression of AXIN pathway components. Any of the aforementioned assays may be used in this cell-based format. Candidate modulators are typically added to the cell media but may also be injected into cells or delivered by any other efficacious means.

Animal Assays

A variety of non-human animal models of normal or defective AXIN pathway may be used to test candidate MAX modulators. Models for defective AXIN pathway typically use genetically modified animals that have been engineered to mis-express (*e.g.*, over-express or lack expression in) genes involved in the AXIN pathway. Assays generally require systemic delivery of the candidate modulators, such as by oral administration, injection, etc.

In a preferred embodiment, AXIN pathway activity is assessed by monitoring neovascularization and angiogenesis. Animal models with defective and normal AXIN are used to test the candidate modulator's affect on MAX in Matrigel® assays. Matrigel®

is an extract of basement membrane proteins, and is composed primarily of laminin, collagen IV, and heparin sulfate proteoglycan. It is provided as a sterile liquid at 4°C, but rapidly forms a solid gel at 37°C. Liquid Matrigel® is mixed with various angiogenic agents, such as bFGF and VEGF, or with human tumor cells which over-express the
5 MAX. The mixture is then injected subcutaneously(SC) into female athymic nude mice (Taconic, Germantown, NY) to support an intense vascular response. Mice with Matrigel® pellets may be dosed via oral (PO), intraperitoneal (IP), or intravenous (IV) routes with the candidate modulator. Mice are euthanized 5 - 12 days post-injection, and the Matrigel® pellet is harvested for hemoglobin analysis (Sigma plasma hemoglobin kit).
10 Hemoglobin content of the gel is found to correlate the degree of neovascularization in the gel.

In another preferred embodiment, the effect of the candidate modulator on MAX is assessed via tumorigenicity assays. Tumor xenograft assays are known in the art (see, e.g., Ogawa K et al., 2000, *Oncogene* 19:6043-6052). Xenografts are typically implanted
15 SC into female athymic mice, 6-7 week old, as single cell suspensions either from a pre-existing tumor or from *in vitro* culture. The tumors which express the MAX endogenously are injected in the flank, 1×10^5 to 1×10^7 cells per mouse in a volume of 100 µL using a 27gauge needle. Mice are then ear tagged and tumors are measured twice weekly. Candidate modulator treatment is initiated on the day the mean tumor weight reaches 100
20 mg. Candidate modulator is delivered IV, SC, IP, or PO by bolus administration. Depending upon the pharmacokinetics of each unique candidate modulator, dosing can be performed multiple times per day. The tumor weight is assessed by measuring perpendicular diameters with a caliper and calculated by multiplying the measurements of diameters in two dimensions. At the end of the experiment, the excised tumors maybe
25 utilized for biomarker identification or further analyses. For immunohistochemistry staining, xenograft tumors are fixed in 4% paraformaldehyde, 0.1M phosphate, pH 7.2, for 6 hours at 4°C, immersed in 30% sucrose in PBS, and rapidly frozen in isopentane cooled with liquid nitrogen.

In another preferred embodiment, tumorigenicity is monitored using a hollow fiber
30 assay, which is described in U.S. Pat No. US 5,698,413. Briefly, the method comprises implanting into a laboratory animal a biocompatible, semi-permeable encapsulation device containing target cells, treating the laboratory animal with a candidate modulating agent, and evaluating the target cells for reaction to the candidate modulator. Implanted cells are generally human cells from a pre-existing tumor or a tumor cell line. After an appropriate

period of time, generally around six days, the implanted samples are harvested for evaluation of the candidate modulator. Tumorigenicity and modulator efficacy may be evaluated by assaying the quantity of viable cells present in the macrocapsule, which can be determined by tests known in the art, for example, MTT dye conversion assay, neutral red dye uptake, trypan blue staining, viable cell counts, the number of colonies formed in soft agar, the capacity of the cells to recover and replicate in vitro, etc.

In another preferred embodiment, a tumorigenicity assay use a transgenic animal, usually a mouse, carrying a dominant oncogene or tumor suppressor gene knockout under the control of tissue specific regulatory sequences; these assays are generally referred to as transgenic tumor assays. In a preferred application, tumor development in the transgenic model is well characterized or is controlled. In an exemplary model, the "RIP1-Tag2" transgene, comprising the SV40 large T-antigen oncogene under control of the insulin gene regulatory regions is expressed in pancreatic beta cells and results in islet cell carcinomas (Hanahan D, 1985, Nature 315:115-122; Parangi S et al, 1996, Proc Natl Acad Sci USA 93: 2002-2007; Bergers G et al, 1999, Science 284:808-812). An "angiogenic switch," occurs at approximately five weeks, as normally quiescent capillaries in a subset of hyperproliferative islets become angiogenic. The RIP1-TAG2 mice die by age 14 weeks. Candidate modulators may be administered at a variety of stages, including just prior to the angiogenic switch (e.g., for a model of tumor prevention), during the growth of small tumors (e.g., for a model of intervention), or during the growth of large and/or invasive tumors (e.g., for a model of regression). Tumorigenicity and modulator efficacy can be evaluating life-span extension and/or tumor characteristics, including number of tumors, tumor size, tumor morphology, vessel density, apoptotic index, etc.

25 **Diagnostic and therapeutic uses**

Specific MAX-modulating agents are useful in a variety of diagnostic and therapeutic applications where disease or disease prognosis is related to defects in the AXIN pathway, such as angiogenic, apoptotic, or cell proliferation disorders. Accordingly, the invention also provides methods for modulating the AXIN pathway in a cell, preferably a cell pre-determined to have defective or impaired AXIN function (e.g. due to overexpression, underexpression, or misexpression of AXIN, or due to gene mutations), comprising the step of administering an agent to the cell that specifically modulates MAX activity. Preferably, the modulating agent produces a detectable phenotypic change in the cell indicating that the AXIN function is restored. The phrase

“function is restored”, and equivalents, as used herein, means that the desired phenotype is achieved, or is brought closer to normal compared to untreated cells. For example, with restored AXIN function, cell proliferation and/or progression through cell cycle may normalize, or be brought closer to normal relative to untreated cells. The invention also provides methods for treating disorders or disease associated with impaired AXIN function by administering a therapeutically effective amount of a MAX -modulating agent that modulates the AXIN pathway. The invention further provides methods for modulating MAX function in a cell, preferably a cell pre-determined to have defective or impaired MAX function, by administering a MAX -modulating agent. Additionally, the invention provides a method for treating disorders or disease associated with impaired MAX function by administering a therapeutically effective amount of a MAX -modulating agent.

The discovery that MAX is implicated in AXIN pathway provides for a variety of methods that can be employed for the diagnostic and prognostic evaluation of diseases and disorders involving defects in the AXIN pathway and for the identification of subjects having a predisposition to such diseases and disorders.

Various expression analysis methods can be used to diagnose whether MAX expression occurs in a particular sample, including Northern blotting, slot blotting, ribonuclease protection, quantitative RT-PCR, and microarray analysis. (*e.g.*, Current Protocols in Molecular Biology (1994) Ausubel FM *et al.*, eds., John Wiley & Sons, Inc., chapter 4; Freeman WM *et al.*, Biotechniques (1999) 26:112-125; Kallioniemi OP, Ann Med 2001, 33:142-147; Blohm and Guiseppi-Elie, Curr Opin Biotechnol 2001, 12:41-47). Tissues having a disease or disorder implicating defective AXIN signaling that express a MAX, are identified as amenable to treatment with a MAX modulating agent. In a preferred application, the AXIN defective tissue overexpresses a MAX relative to normal tissue. For example, a Northern blot analysis of mRNA from tumor and normal cell lines, or from tumor and matching normal tissue samples from the same patient, using full or partial MAX cDNA sequences as probes, can determine whether particular tumors express or overexpress MAX. Alternatively, the TaqMan® is used for quantitative RT-PCR analysis of MAX expression in cell lines, normal tissues and tumor samples (PE Applied Biosystems).

Various other diagnostic methods may be performed, for example, utilizing reagents such as the MAX oligonucleotides, and antibodies directed against a MAX, as described above for: (1) the detection of the presence of MAX gene mutations, or the

detection of either over- or under-expression of MAX mRNA relative to the non-disorder state; (2) the detection of either an over- or an under-abundance of MAX gene product relative to the non-disorder state; and (3) the detection of perturbations or abnormalities in the signal transduction pathway mediated by MAX.

5 Thus, in a specific embodiment, the invention is drawn to a method for diagnosing a disease or disorder in a patient that is associated with alterations in MAX expression, the method comprising: a) obtaining a biological sample from the patient; b) contacting the sample with a probe for MAX expression; c) comparing results from step (b) with a control; and d) determining whether step (c) indicates a likelihood of the disease or
10 disorder. Preferably, the disease is cancer. The probe may be either DNA or protein, including an antibody.

EXAMPLES

15 The following experimental section and examples are offered by way of illustration and not by way of limitation.

I. C. elegans AXIN screen

20 We have found that the temperature-sensitive, reduction-of-function *pry-1* mutant *mu38* grown at 15°C produces a ruptured vulva (Rvl) phenotype by which about 95% of animals become eviscerated and die at the L4 molt. The *pry-1* Rvl mutant phenotype is suppressed by loss-of-function mutations in the beta-catenin ortholog *bar-1* and the TCF ortholog *pop-1*. The Rvl phenotype can also be generated by gain-of-function mutations in *bar-1*/beta-catenin that eliminate the consensus GSK3-beta phosphorylation sites and are predicted to prevent Axin-mediated degradation of BAR-1.

25 We designed a genetic screen to identify genes in addition to *bar-1*/beta-catenin and *pop-1*/TCF that act positively in beta-catenin signaling and, when inactivated, suppress the Rvl mutant phenotype of *pry-1*/Axin. The function of individual genes was inactivated by RNAi in *pry-1* (*mu38*) L1 larvae, and suppression of the Rvl phenotype was scored as a statistically significant increase in the proportion of larvae that survived to
30 adulthood without rupturing. Suppressor genes were subsequently counterscreened to eliminate those that appeared to suppress the *pry-1* mutant non-specifically, rather than those that specifically functioned in beta-catenin signaling. Suppressor genes that did not block vulva formation in a wildtype background, and that did not suppress the Rvl phenotype of two mutations in genes unrelated to beta-catenin signaling (*lin-1*/Ets and *daf-*

18/PTEN) were considered to be specific *pyr-1*/Axin suppressors. These suppressor genes, when inactivated, likely suppress beta-catenin's inappropriate transcriptional activation of target genes and, therefore, may be relevant for cancer therapy.

5 II. Analysis of Table 1

BLAST analysis (Altschul et al., *supra*) was employed to identify orthologs of *C. elegans* modifiers. The columns "MAX symbol", and "MAX name aliases" provide a symbol and the known name abbreviations for the Targets, where available, from Genbank. "MAX RefSeq_NA or GI_NA", "MAX GI_AA", "MAX NAME", and "MAX Description" provide the reference DNA sequences for the MAXs as available from National Center for Biology Information (NCBI), MAX protein Genbank identifier number (GI#), MAX name, and MAX description, all available from Genbank, respectively. The length of each amino acid is in the "MAX Protein Length" column.

Names and Protein sequences of *C. elegans* modifiers of AXIN from screen (Example I), are represented in the "Modifier Name" and "Modifier GI_AA" column by GI#, respectively.

Table 1

MAX symbol	MAX name aliases	MAX RefSeq_NA or GI_NA	NA SE Q ID NO	MAX GI_AA	AA SE Q ID NO	MAX NAME	MAX DESCRIPTION	MAX PROTEIN LENGTH	MODIFIER NAME	MODIFIER GI_AA
MBTP S2	S2P, S2P protein, membrane-bound transcription factor protease, site 2	NM_015884.1	1	7706693	8	membrane-bound transcription factor protease, site 2	metalloendopeptidase	519	Y56A2A.2	17556258
STK12	AUR1, AIK2, AIM1, ARK2, IPL1, AIM-1, STK-1, serine/threonine kinase 12	NM_004217.1	2	4759178	9	serine/threonine kinase 12	protein kinase; protein kinase; protein serine/threonine kinase; protein serine/threonine kinase	344	B0207.4	17505246

STK13	AUR3, AIE2, AIK3, AIE1_m, serine/threonine kinase 13 (aurora/IPL1-like)	NM_003160.1 XM_030228.2	3	4507273	10	serine/threonine kinase 13 (aurora/IPL1-like)	protein kinase; protein serine/threonine kinase	275	B0207.4	17505246
STK6	AIK, BTAK, STK15, aurora-A, serine/threonine protein kinase-6, serine/threonine kinase 6	NM_003158.1 NM_003600.1	4	4507275	11	serine/threonine kinase 6	protein kinase; protein serine/threonine kinase	403	B0207.4	17505246
BUB1B	BUBR1, Bub1A, MAD3L, hBUBR1, budding uninhibited by benzimidazoles 1 (yeast homolog), beta, BUB1 budding uninhibited by benzimidazoles 1 homolog beta (yeast)	NM_001211.3 XM_031603.1	5	5729750	12	BUB1 budding uninhibited by benzimidazoles 1 homolog beta (yeast)	BUB1 budding uninhibited by benzimidazole s 1 homolog beta, protein kinase, acts in the mitotic spindle checkpoint, detects kinetochore tension; variants of the corresponding gene are associated with adult T cell leukemia and colorectal cancer	1050	R06C7.8	17508581

BUB1	hBUB1, putative serine/threonine-protein kinase, budding uninhibited by benzimidazoles 1 (yeast homolog), BUB1 budding uninhibited by benzimidazoles 1 homolog (yeast)	NM_004336.1	6	4757878	13	BUB1 budding uninhibited by benzimidazoles 1 homolog (yeast)	Budding uninhibited by benzimidazoles 1 homolog, a spindle assembly checkpoint protein that may sense kinetochore tension; mutations are associated with lung cancer, adult T cell leukemia, and chromosome instability in colorectal cancer cell lines	10858	R06C7.8	17508581
RBBP6		NM_006910.1 XM_056260.4	7	5902044	14	retinoblastoma binding protein 6	Retinoblastoma-binding protein 6, contains a serine-arginine (SR) rich region, and a central region with many different short, repetitive sequences, forms a complex with p53 (TP53) and hypophosphorylated RB1, may function as a pre-mRNA splicing factor	948	F36F2.3	17507217

III. High-Throughput In Vitro Fluorescence Polarization Assay

Fluorescently-labeled MAX peptide/substrate are added to each well of a 96-well microtiter plate, along with a test agent in a test buffer (10 mM HEPES, 10 mM NaCl, 6 mM magnesium chloride, pH 7.6). Changes in fluorescence polarization, determined by using a Fluorolite FPM-2 Fluorescence Polarization Microtiter System (Dynatech Laboratories, Inc), relative to control values indicates the test compound is a candidate modifier of MAX activity.

IV. High-Throughput In Vitro Binding Assay.

³³P-labeled MAX peptide is added in an assay buffer (100 mM KCl, 20 mM HEPES pH 7.6, 1 mM MgCl₂, 1% glycerol, 0.5% NP-40, 50 mM beta-mercaptoethanol, 1 mg/ml BSA, cocktail of protease inhibitors) along with a test agent to the wells of a Neutralite-avidin coated assay plate and incubated at 25°C for 1 hour. Biotinylated substrate is then added to each well and incubated for 1 hour. Reactions are stopped by washing with PBS, and counted in a scintillation counter. Test agents that cause a difference in activity relative to control without test agent are identified as candidate AXIN modulating agents.

10

V. Immunoprecipitations and Immunoblotting

For coprecipitation of transfected proteins, 3×10^6 appropriate recombinant cells containing the MAX proteins are plated on 10-cm dishes and transfected on the following day with expression constructs. The total amount of DNA is kept constant in each transfection by adding empty vector. After 24 h, cells are collected, washed once with phosphate-buffered saline and lysed for 20 min on ice in 1 ml of lysis buffer containing 50 mM Hepes, pH 7.9, 250 mM NaCl, 20 mM -glycerophosphate, 1 mM sodium orthovanadate, 5 mM p-nitrophenyl phosphate, 2 mM dithiothreitol, protease inhibitors (complete, Roche Molecular Biochemicals), and 1% Nonidet P-40. Cellular debris is removed by centrifugation twice at $15,000 \times g$ for 15 min. The cell lysate is incubated with 25 μ l of M2 beads (Sigma) for 2 h at 4 °C with gentle rocking.

After extensive washing with lysis buffer, proteins bound to the beads are solubilized by boiling in SDS sample buffer, fractionated by SDS-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membrane and blotted with the indicated antibodies. The reactive bands are visualized with horseradish peroxidase coupled to the appropriate secondary antibodies and the enhanced chemiluminescence (ECL) Western blotting detection system (Amersham Pharmacia Biotech).

VI. Kinase assay

A purified or partially purified MAX is diluted in a suitable reaction buffer, e.g., 50 mM Hepes, pH 7.5, containing magnesium chloride or manganese chloride (1-20 mM) and a peptide or polypeptide substrate, such as myelin basic protein or casein (1-10 μ g/ml). The final concentration of the kinase is 1-20 nM. The enzyme reaction is conducted in microtiter plates to facilitate optimization of reaction conditions by

increasing assay throughput. A 96-well microtiter plate is employed using a final volume 30-100 μ l. The reaction is initiated by the addition of ^{33}P -gamma-ATP (0.5 $\mu\text{Ci/ml}$) and incubated for 0.5 to 3 hours at room temperature. Negative controls are provided by the addition of EDTA, which chelates the divalent cation (Mg^{2+} or Mn^{2+}) required for enzymatic activity. Following the incubation, the enzyme reaction is quenched using EDTA. Samples of the reaction are transferred to a 96-well glass fiber filter plate (MultiScreen, Millipore). The filters are subsequently washed with phosphate-buffered saline, dilute phosphoric acid (0.5%) or other suitable medium to remove excess radiolabeled ATP. Scintillation cocktail is added to the filter plate and the incorporated radioactivity is quantitated by scintillation counting (Wallac/Perkin Elmer). Activity is defined by the amount of radioactivity detected following subtraction of the negative control reaction value (EDTA quench).

VII. Expression analysis

All cell lines used in the following experiments are NCI (National Cancer Institute) lines, and are available from ATCC (American Type Culture Collection, Manassas, VA 20110-2209). Normal and tumor tissues are obtained from Impath, UC Davis, Clontech, Stratagene, Ardaïs, Genome Collaborative, and Ambion.

TaqMan analysis is used to assess expression levels of the disclosed genes in various samples.

RNA is extracted from each tissue sample using Qiagen (Valencia, CA) RNeasy kits, following manufacturer's protocols, to a final concentration of 50ng/ μ l. Single stranded cDNA is then synthesized by reverse transcribing the RNA samples using random hexamers and 500ng of total RNA per reaction, following protocol 4304965 of Applied Biosystems (Foster City, CA).

Primers for expression analysis using TaqMan assay (Applied Biosystems, Foster City, CA) are prepared according to the TaqMan protocols, and the following criteria: a) primer pairs are designed to span introns to eliminate genomic contamination, and b) each primer pair produced only one product. Expression analysis is performed using a 7900HT instrument.

Taqman reactions are carried out following manufacturer's protocols, in 25 μ l total volume for 96-well plates and 10 μ l total volume for 384-well plates, using 300nM primer and 250 nM probe, and approximately 25ng of cDNA. The standard curve for result analysis is prepared using a universal pool of human cDNA samples, which is a mixture of

cDNAs from a wide variety of tissues so that the chance that a target will be present in appreciable amounts is good. The raw data are normalized using 18S rRNA (universally expressed in all tissues and cells).

For each expression analysis, tumor tissue samples are compared with matched
5 normal tissues from the same patient. A gene is considered overexpressed in a tumor when the level of expression of the gene is 2 fold or higher in the tumor compared with its matched normal sample. In cases where normal tissue is not available, a universal pool of cDNA samples is used instead. In these cases, a gene is considered overexpressed in a tumor sample when the difference of expression levels between a tumor sample and the
10 average of all normal samples from the same tissue type is greater than 2 times the standard deviation of all normal samples (i.e., $\text{Tumor} - \text{average}(\text{all normal samples}) > 2 \times \text{STDEV}(\text{all normal samples})$).

A modulator identified by an assay described herein can be further validated for therapeutic effect by administration to a tumor in which the gene is overexpressed. A
15 decrease in tumor growth confirms therapeutic utility of the modulator. Prior to treating a patient with the modulator, the likelihood that the patient will respond to treatment can be diagnosed by obtaining a tumor sample from the patient, and assaying for expression of the gene targeted by the modulator. The expression data for the gene(s) can also be used as a diagnostic marker for disease progression. The assay can be performed by expression
20 analysis as described above, by antibody directed to the gene target, or by any other available detection method.

WHAT IS CLAIMED IS:

1. A method of identifying a candidate AXIN pathway modulating agent, said method comprising the steps of:
 - 5 (a) providing an assay system comprising a MAX polypeptide or nucleic acid;
 - (b) contacting the assay system with a test agent under conditions whereby, but for the presence of the test agent, the system provides a reference activity; and
 - (c) detecting a test agent-biased activity of the assay system, wherein a difference between the test agent-biased activity and the reference activity identifies the test agent as
 - 10 a candidate AXIN pathway modulating agent.
2. The method of Claim 1 wherein the assay system comprises cultured cells that express the MAX polypeptide.
- 15 3. The method of Claim 2 wherein the cultured cells additionally have defective AXIN function.
4. The method of Claim 1 wherein the assay system includes a screening assay comprising a MAX polypeptide, and the candidate test agent is a small molecule
- 20 modulator.
5. The method of Claim 4 wherein the assay is a binding assay.
6. The method of Claim 1 wherein the assay system is selected from the group consisting
- 25 of an apoptosis assay system, a cell proliferation assay system, an angiogenesis assay system, and a hypoxic induction assay system.
7. The method of Claim 1 wherein the assay system includes a binding assay comprising a MAX polypeptide and the candidate test agent is an antibody.
- 30 8. The method of Claim 1 wherein the assay system includes an expression assay comprising a MAX nucleic acid and the candidate test agent is a nucleic acid modulator.
9. The method of Claim 8 wherein the nucleic acid modulator is an antisense oligomer.

10. The method of Claim 8 wherein the nucleic acid modulator is a PMO.

11. The method of Claim 1 additionally comprising:

5 (d) administering the candidate AXIN pathway modulating agent identified in (c) to a model system comprising cells defective in AXIN function and, detecting a phenotypic change in the model system that indicates that the AXIN function is restored.

12. The method of Claim 11 wherein the model system is a mouse model with defective AXIN function.

10

13. A method for modulating a AXIN pathway of a cell comprising contacting a cell defective in AXIN function with a candidate modulator that specifically binds to a MAX polypeptide, whereby AXIN function is restored.

15 14. The method of Claim 13 wherein the candidate modulator is administered to a vertebrate animal predetermined to have a disease or disorder resulting from a defect in AXIN function.

20 15. The method of Claim 13 wherein the candidate modulator is selected from the group consisting of an antibody and a small molecule.

16. The method of Claim 1, comprising the additional steps of:

(e) providing a secondary assay system comprising cultured cells or a non-human animal expressing MAX ,

25 (f) contacting the secondary assay system with the test agent of (b) or an agent derived therefrom under conditions whereby, but for the presence of the test agent or agent derived therefrom, the system provides a reference activity; and

(g) detecting an agent-biased activity of the second assay system,
wherein a difference between the agent-biased activity and the reference activity of
30 the second assay system confirms the test agent or agent derived therefrom as a candidate AXIN pathway modulating agent,

and wherein the second assay detects an agent-biased change in the AXIN pathway.

17. The method of Claim 16 wherein the secondary assay system comprises cultured cells.
18. The method of Claim 16 wherein the secondary assay system comprises a non-human
5 animal.
19. The method of Claim 18 wherein the non-human animal mis-expresses a AXIN pathway gene.
- 10 20. A method of modulating AXIN pathway in a mammalian cell comprising contacting the cell with an agent that specifically binds a MAX polypeptide or nucleic acid.
21. The method of Claim 20 wherein the agent is administered to a mammalian animal predetermined to have a pathology associated with the AXIN pathway.
- 15 22. The method of Claim 20 wherein the agent is a small molecule modulator, a nucleic acid modulator, or an antibody.
23. A method for diagnosing a disease in a patient comprising:
- 20 (a) obtaining a biological sample from the patient;
(b) contacting the sample with a probe for MAX expression;
(c) comparing results from step (b) with a control;
(d) determining whether step (c) indicates a likelihood of disease.
- 25 24. The method of claim 23 wherein said disease is cancer.

SEQUENCE LISTING

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<120> MAXS AS MODIFIERS OF THE AXIN PATHWAY AND METHODS OF USE

<130> EX03-051C-PC

<150> US 60/401,534

<151> 2002-08-07

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gattgagcaa gtgcatgact gtgaaatcat tcatggagac attaaaccag acaatttcac	2820
acttggaac ggatttttgg aacaggatga tgaagatgat ttatctgctg gcttggcact	2880
gattgacctg ggtcagagta tagatatgaa actttttcca aaaggaacta tattcacagc	2940

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gaaagtgaaa aatgaaggag gagagtgtaa gcctgaaggt ctttttagaa ggcttcctca 3120
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caagattagg gccctacgta ataggctaata tgtactgctc ttagaatgta agcgttcacg 3300
aaaataaaat ttggatatag acagtcctta aaaatcacac tgtaaataatg aatctgctca 3360
ctttaaacct gttttttttt catttattgt ttatgtaaata gtttggttaa aataaatccc 3420
atggaatatt tccatgtaaa aaaaaa 3446

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<210> 7
<211> 2994
<212> DNA
<213> Homo sapiens

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aggattaaaa agagcactgg aattcccaga agttttcatg atggaagtga aagatcctaa 180
tatgaaaggt gcaatgctta ccaacactgg aaaatatgca ataccaacta tagatgcaga 240
agcatatgca attgggaaga aagagaaacc tcccttctta ccagaggagc catcttcttc 300
ctcagaagaa gatgatccta tcccagatga attgttgtgt ctcatctgca aggatattat 360
gactgatgct gttgtgattc cctgctgtgg aaacagttac tgtgatgaat gtataagaac 420
agcactcctg gaatcagatg agcacacatg tccgacgtgt catcaaaatg atgtttctcc 480
tgatgcttta attgccaata aattttttacg acaggctgta aataacttca aaaatgaaac 540
tggctatata aaaagactac gaaaacagtt acctcctcca ccacccccaa taccacctcc 600
gagaccactg attcagagga acctacaacc tctgatgaga tctccgatat caagacaaca 660
agatcctctt atgattccag tgacatcttc atcaactcac ccagctccgt ctatatcttc 720
attaacttct aatcagtctt ccttggcccc tcctgtgtct ggaaatccgt cttctgctcc 780
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ctcaaaggga acctcctcaa ttgcaattac cgctcttatg gaagagaagg gttaccaggt 960
gcctgttctt ggaaccccat ctttgcttgg acagtcatta ttgcatggac agttgatccc 1020
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acattccaac	aaacttggct	atctggtttc	tccaccacaa	caaattagaa	gaggggagag	1140
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gcctcctccc	catacacttc	ctctccctcc	gggtgttctt	cctccacagt	tttctcctca	1320
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agctcctgcc	aatttatcaa	caccttgggt	atcatcagga	gtgcagacag	ctcattcaaa	1440
taccatccca	acaacacaag	caccaccttt	gtccagggaa	gaattctata	gagagcagcg	1500
acgactaaaa	gaagaggaaa	agaaaaagtc	caagctagat	gagtttataa	atgattttgc	1560
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tcattcacga	tcaagatctc	ctcaagcgtt	taggggacag	tctcctaata	aacgtaatgt	1920
acctcaaggg	gaaacagaac	gtgaatat	taatagatac	agagaagttc	caccaccata	1980
tgacatgaaa	gcatattatg	ggagaagtgt	tgactttaga	gaccatttg	aaaaagaacg	2040
ctaccgagaa	tgggagagaa	aatatagaga	gtggtatgaa	aaatattata	aaggttatgc	2100
tgctggagca	cagcctagac	cctcagcaaa	tagagagaac	ttttctccag	agagattttt	2160
gccacttaac	atcaggaatt	ctcccttcac	aagaggccgc	agagaagact	atggttggtg	2220
gcaaagtcac	agaagtcgaa	acataggtag	caactatcca	gaaaagcttt	cagcaagaga	2280
tggtcacac	cagaaggata	atacaaagtc	aaaagagaag	gagagtga	acgctccagg	2340
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gggttttctg	aaccagagt	tattagagac	ttctaggaaa	tcaagagaac	ctacaggtgt	2460
tgaagaaaat	aaaacagact	cattgtttgt	tctcccaagt	agagatgatg	ccacacctgt	2520
tagagatgaa	ccaatggatg	cagaatcaat	cacttttaaa	tcagtgtctg	aaaaagacaa	2580
gagagaaagg	gataaaccac	aagcaaagg	tgataaaacc	aaacggaaga	atgatggatc	2640
tgctgtgtcc	aaaaaagaaa	atattgtaaa	acctgctaaa	ggaccccaag	aaaaagtaga	2700
tggagacgtg	agagatctcc	tcgatctgaa	cctccaatta	aaaaagccaa	agaggagact	2760
ccgaagactg	acaatactaa	atcatcatct	tcctctcaga	aggatgaaaa	aatcactgga	2820
acccccagaa	aagctcactc	taaatcagca	aaagacacca	agaaacaaaa	ccagtcaaag	2880
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aagaaaaggc caagaagcct aatgagaaaa acaaaccact tgataataag ggag 2994

<210> 8
 <211> 519
 <212> PRT
 <213> Homo sapiens

<400> 8

Met Ile Pro Val Ser Leu Val Val Val Val Val Gly Gly Trp Thr Val
 1 5 10 15

Val Tyr Leu Thr Asp Leu Val Leu Lys Ser Ser Val Tyr Phe Lys His
 20 25 30

Ser Tyr Glu Asp Trp Leu Glu Asn Asn Gly Leu Ser Ile Ser Pro Phe
 35 40 45

His Ile Arg Trp Gln Thr Ala Val Phe Asn Arg Ala Phe Tyr Ser Trp
 50 55 60

Gly Arg Arg Lys Ala Arg Met Leu Tyr Gln Trp Phe Asn Phe Gly Met
 65 70 75 80

Val Phe Gly Val Ile Ala Met Phe Ser Ser Phe Phe Leu Leu Gly Lys
 85 90 95

Thr Leu Met Gln Thr Leu Ala Gln Met Met Ala Asp Ser Pro Ser Ser
 100 105 110

Tyr Ser Ser Ser Ser Ser Ser Ser Ser Ser Ser Ser Ser Ser Ser Ser
 115 120 125

Ser Ser Ser Ser Ser Ser Ser Ser Leu His Asn Glu Gln Val Leu Gln
 130 135 140

Val Val Val Pro Gly Ile Asn Leu Pro Val Asn Gln Leu Thr Tyr Phe
 145 150 155 160

Phe Thr Ala Val Leu Ile Ser Gly Val Val His Glu Ile Gly His Gly
 165 170 175

Ile Ala Ala Ile Arg Glu Gln Val Arg Phe Asn Gly Phe Gly Ile Phe
 180 185 190

Leu Phe Ile Ile Tyr Pro Gly Ala Phe Val Asp Leu Phe Thr Thr His
 195 200 205

Leu Gln Leu Ile Ser Pro Val Gln Gln Leu Arg Ile Phe Cys Ala Gly
 210 215 220

Ile Trp His Asn Phe Val Leu Ala Leu Leu Gly Ile Leu Ala Leu Val
 225 230 235 240

Leu Leu Pro Val Ile Leu Leu Pro Phe Tyr Tyr Thr Gly Val Gly Val
 245 250 255

Leu Ile Thr Glu Val Ala Glu Asp Ser Pro Ala Ile Gly Pro Arg Gly
 260 265 270

Leu Phe Val Gly Asp Leu Val Thr His Leu Gln Asp Cys Pro Val Thr
 275 280 285

Asn Val Gln Asp Trp Asn Glu Cys Leu Asp Thr Ile Ala Tyr Glu Pro
 290 295 300

Gln Ile Gly Tyr Cys Ile Ser Ala Ser Thr Leu Gln Gln Leu Ser Phe
 305 310 315 320

Pro Val Arg Ala Tyr Lys Arg Leu Asp Gly Ser Thr Glu Cys Cys Asn
 325 330 335

Asn His Ser Leu Thr Asp Val Cys Phe Ser Tyr Arg Asn Asn Phe Asn
 340 345 350

Lys Arg Leu His Thr Cys Leu Pro Ala Arg Lys Ala Val Glu Ala Thr
 355 360 365

Gln Val Cys Arg Thr Asn Lys Asp Cys Lys Lys Ser Ser Ser Ser Ser
 370 375 380

Phe Cys Ile Ile Pro Ser Leu Glu Thr His Thr Arg Leu Ile Lys Val
 385 390 395 400

Lys His Pro Pro Gln Ile Asp Met Leu Tyr Val Gly His Pro Leu His
 405 410 415

Leu His Tyr Thr Val Ser Ile Thr Ser Phe Ile Pro Arg Phe Asn Phe
 420 425 430

Leu Ser Ile Asp Leu Pro Val Val Val Glu Thr Phe Val Lys Tyr Leu
 435 440 445

Ile Ser Leu Ser Gly Ala Leu Ala Ile Val Asn Ala Val Pro Cys Phe
 450 455 460

Ala Leu Asp Gly Gln Trp Ile Leu Asn Ser Phe Leu Asp Ala Thr Leu
465 470 475 480

Thr Ser Val Ile Gly Asp Asn Asp Val Lys Asp Leu Ile Gly Phe Phe
485 490 495

Ile Leu Leu Gly Gly Ser Val Leu Leu Ala Ala Asn Val Thr Leu Gly
500 505 510

Leu Trp Met Val Thr Ala Arg
515

<210> 9
<211> 344
<212> PRT
<213> Homo sapiens

<400> 9

Met Ala Gln Lys Glu Asn Ser Tyr Pro Trp Pro Tyr Gly Arg Gln Thr
1 5 10 15

Ala Pro Ser Gly Leu Ser Thr Leu Pro Gln Arg Val Leu Arg Lys Glu
20 25 30

Pro Val Thr Pro Ser Ala Leu Val Leu Met Ser Arg Ser Asn Val Gln
35 40 45

Pro Thr Ala Ala Pro Gly Gln Lys Val Met Glu Asn Ser Ser Gly Thr
50 55 60

Pro Asp Ile Leu Thr Arg His Phe Thr Ile Asp Asp Phe Glu Ile Gly
65 70 75 80

Arg Pro Leu Gly Lys Gly Lys Phe Gly Asn Val Tyr Leu Ala Arg Glu
85 90 95

Lys Lys Ser His Phe Ile Val Ala Leu Lys Val Leu Phe Lys Ser Gln
100 105 110

Ile Glu Lys Glu Gly Val Glu His Gln Leu Arg Arg Glu Ile Glu Ile
115 120 125

Gln Ala His Leu His His Pro Asn Ile Leu Arg Leu Tyr Asn Tyr Phe
130 135 140

Tyr Asp Arg Arg Arg Ile Tyr Leu Ile Leu Glu Tyr Ala Pro Arg Gly

Met Arg Arg Leu Thr Val Asp Asp Phe Glu Ile Gly Arg Pro Leu Gly
1 5 10 15

Lys Gly Lys Phe Gly Asn Val Tyr Leu Ala Arg Leu Lys Glu Ser His
 20 25 30

Phe Ile Val Ala Leu Lys Val Leu Phe Lys Ser Gln Ile Glu Lys Glu
 35 40 45

Gly Leu Glu His Gln Leu Arg Arg Glu Ile Glu Ile Gln Ala His Leu
 50 55 60

Gln His Pro Asn Ile Leu Arg Leu Tyr Asn Tyr Phe His Asp Ala Arg
 65 70 75 80

Arg Val Tyr Leu Ile Leu Glu Tyr Ala Pro Arg Gly Glu Leu Tyr Lys
 85 90 95

Glu Leu Gln Lys Ser Glu Lys Leu Asp Glu Gln Arg Thr Ala Thr Ile
 100 105 110

Ile Glu Glu Val Ala Asp Ala Leu Thr Tyr Cys His Asp Lys Lys Val
 115 120 125

Ile His Arg Asp Ile Lys Pro Glu Asn Leu Leu Leu Gly Phe Arg Gly
 130 135 140

Glu Val Lys Ile Ala Asp Phe Gly Trp Ser Val His Thr Pro Leu Pro
 145 150 155 160

Glu Arg Lys Thr Met Cys Gly Thr Leu Asp Tyr Leu Pro Pro Glu Met
 165 170 175

Ile Glu Gly Arg Thr Tyr Asp Glu Lys Val Asp Leu Trp Cys Ile Gly
 180 185 190

Val Leu Cys Tyr Glu Leu Leu Val Gly Tyr Pro Pro Phe Glu Ser Ala
 195 200 205

Ser His Ser Glu Thr Tyr Arg Arg Ile Leu Lys Val Asp Val Arg Phe
 210 215 220

Pro Leu Ser Met Pro Leu Gly Ala Arg Asp Leu Ile Ser Arg Leu Leu
 225 230 235 240

Arg Tyr Gln Pro Leu Glu Arg Leu Pro Leu Ala Gln Ile Leu Lys His
 245 250 255

Pro Trp Val Gln Ala His Ser Arg Arg Val Leu Pro Pro Cys Ala Gln
 260 265 270

Met Ala Ser
275

<210> 11
<211> 403
<212> PRT
<213> Homo sapiens

<400> 11

Met Asp Arg Ser Lys Glu Asn Cys Ile Ser Gly Pro Val Lys Ala Thr
1 5 10 15

Ala Pro Val Gly Gly Pro Lys Arg Val Leu Val Thr Gln Gln Ile Pro
20 25 30

Cys Gln Asn Pro Leu Pro Val Asn Ser Gly Gln Ala Gln Arg Val Leu
35 40 45

Cys Pro Ser Asn Ser Ser Gln Arg Val Pro Leu Gln Ala Gln Lys Leu
50 55 60

Val Ser Ser His Lys Pro Val Gln Asn Gln Lys Gln Lys Gln Leu Gln
65 70 75 80

Ala Thr Ser Val Pro His Pro Val Ser Arg Pro Leu Asn Asn Thr Gln
85 90 95

Lys Ser Lys Gln Pro Leu Pro Ser Ala Pro Glu Asn Asn Pro Glu Glu
100 105 110

Glu Leu Ala Ser Lys Gln Lys Asn Glu Glu Ser Lys Lys Arg Gln Trp
115 120 125

Ala Leu Glu Asp Phe Glu Ile Gly Arg Pro Leu Gly Lys Gly Lys Phe
130 135 140

Gly Asn Val Tyr Leu Ala Arg Glu Lys Gln Ser Lys Phe Ile Leu Ala
145 150 155 160

Leu Lys Val Leu Phe Lys Ala Gln Leu Glu Lys Ala Gly Val Glu His
165 170 175

Gln Leu Arg Arg Glu Val Glu Ile Gln Ser His Leu Arg His Pro Asn
180 185 190

Ile Leu Arg Leu Tyr Gly Tyr Phe His Asp Ala Thr Arg Val Tyr Leu

195	200	205
Ile Leu Glu Tyr Ala Pro Leu Gly Thr Val Tyr Arg Glu Leu Gln Lys 210	215	220
Leu Ser Lys Phe Asp Glu Gln Arg Thr Ala Thr Tyr Ile Thr Glu Leu 225	230	235 240
Ala Asn Ala Leu Ser Tyr Cys His Ser Lys Arg Val Ile His Arg Asp 245	250	255
Ile Lys Pro Glu Asn Leu Leu Leu Gly Ser Ala Gly Glu Leu Lys Ile 260	265	270
Ala Asp Phe Gly Trp Ser Val His Ala Pro Ser Ser Arg Arg Thr Thr 275	280	285
Leu Cys Gly Thr Leu Asp Tyr Leu Pro Pro Glu Met Ile Glu Gly Arg 290	295	300
Met His Asp Glu Lys Val Asp Leu Trp Ser Leu Gly Val Leu Cys Tyr 305	310	315 320
Glu Phe Leu Val Gly Lys Pro Pro Phe Glu Ala Asn Thr Tyr Gln Glu 325	330	335
Thr Tyr Lys Arg Ile Ser Arg Val Glu Phe Thr Phe Pro Asp Phe Val 340	345	350
Thr Glu Gly Ala Arg Asp Leu Ile Ser Arg Leu Leu Lys His Asn Pro 355	360	365
Ser Gln Arg Pro Met Leu Arg Glu Val Leu Glu His Pro Trp Ile Thr 370	375	380
Ala Asn Ser Ser Lys Pro Ser Asn Cys Gln Asn Lys Glu Ser Ala Ser 385	390	395 400
Lys Gln Ser		

<210> 12
 <211> 1050
 <212> PRT
 <213> Homo sapiens
 <400> 12

Met Ala Ala Val Lys Lys Glu Gly Gly Ala Leu Ser Glu Ala Met Ser
 1 5 10 15
 Leu Glu Gly Asp Glu Trp Glu Leu Ser Lys Glu Asn Val Gln Pro Leu
 20 25 30
 Arg Gln Gly Arg Ile Met Ser Thr Leu Gln Gly Ala Leu Ala Gln Glu
 35 40 45
 Ser Ala Cys Asn Asn Thr Leu Gln Gln Gln Lys Arg Ala Phe Glu Tyr
 50 55 60
 Glu Ile Arg Phe Tyr Thr Gly Asn Asp Pro Leu Asp Val Trp Asp Arg
 65 70 75 80
 Tyr Ile Ser Trp Thr Glu Gln Asn Tyr Pro Gln Gly Gly Lys Glu Ser
 85 90 95
 Asn Met Ser Thr Leu Leu Glu Arg Ala Val Glu Ala Leu Gln Gly Glu
 100 105 110
 Lys Arg Tyr Tyr Ser Asp Pro Arg Phe Leu Asn Leu Trp Leu Lys Leu
 115 120 125
 Gly Arg Leu Cys Asn Glu Pro Leu Asp Met Tyr Ser Tyr Leu His Asn
 130 135 140
 Gln Gly Ile Gly Val Ser Leu Ala Gln Phe Tyr Ile Ser Trp Ala Glu
 145 150 155 160
 Glu Tyr Glu Ala Arg Glu Asn Phe Arg Lys Ala Asp Ala Ile Phe Gln
 165 170 175
 Glu Gly Ile Gln Gln Lys Ala Glu Pro Leu Glu Arg Leu Gln Ser Gln
 180 185 190
 His Arg Gln Phe Gln Ala Arg Val Ser Arg Gln Thr Leu Leu Ala Leu
 195 200 205
 Glu Lys Glu Glu Glu Glu Glu Val Phe Glu Ser Ser Val Pro Gln Arg
 210 215 220
 Ser Thr Leu Ala Glu Leu Lys Ser Lys Gly Lys Lys Thr Ala Arg Ala
 225 230 235 240
 Pro Ile Ile Arg Val Gly Gly Ala Leu Lys Ala Pro Ser Gln Asn Arg
 245 250 255

Gly Leu Gln Asn Pro Phe Pro Gln Gln Met Gln Asn Asn Ser Arg Ile
 260 265 270

Thr Val Phe Asp Glu Asn Ala Asp Glu Ala Ser Thr Ala Glu Leu Ser
 275 280 285

Lys Pro Thr Val Gln Pro Trp Ile Ala Pro Pro Met Pro Arg Ala Lys
 290 295 300

Glu Asn Glu Leu Gln Ala Gly Pro Trp Asn Thr Gly Arg Ser Leu Glu
 305 310 315 320

His Arg Pro Arg Gly Asn Thr Ala Ser Leu Ile Ala Val Pro Ala Val
 325 330 335

Leu Pro Ser Phe Thr Pro Tyr Val Glu Glu Thr Ala Gln Gln Pro Val
 340 345 350

Met Thr Pro Cys Lys Ile Glu Pro Ser Ile Asn His Ile Leu Ser Thr
 355 360 365

Arg Lys Pro Gly Lys Glu Glu Gly Asp Pro Leu Gln Arg Val Gln Ser
 370 375 380

His Gln Gln Ala Ser Glu Glu Lys Lys Glu Lys Met Met Tyr Cys Lys
 385 390 395 400

Glu Lys Ile Tyr Ala Gly Val Gly Glu Phe Ser Phe Glu Glu Ile Arg
 405 410 415

Ala Glu Val Phe Arg Lys Lys Leu Lys Glu Gln Arg Glu Ala Glu Leu
 420 425 430

Leu Thr Ser Ala Glu Lys Arg Ala Glu Met Gln Lys Gln Ile Glu Glu
 435 440 445

Met Glu Lys Lys Leu Lys Glu Ile Gln Thr Thr Gln Gln Glu Arg Thr
 450 455 460

Gly Asp Gln Gln Glu Glu Thr Met Pro Thr Lys Glu Thr Thr Lys Leu
 465 470 475 480

Gln Ile Ala Ser Glu Ser Gln Lys Ile Pro Gly Met Thr Leu Ser Ser
 485 490 495

Ser Val Cys Gln Val Asn Cys Cys Ala Arg Glu Thr Ser Leu Ala Glu
 500 505 510

Asn Ile Trp Gln Glu Gln Pro His Ser Lys Gly Pro Ser Val Pro Phe
 515 520 525

Ser Ile Phe Asp Glu Phe Leu Leu Ser Glu Lys Lys Asn Lys Ser Pro
 530 535 540

Pro Ala Asp Pro Pro Arg Val Leu Ala Gln Arg Arg Pro Leu Ala Val
 545 550 555 560

Leu Lys Thr Ser Glu Ser Ile Thr Ser Asn Glu Asp Val Ser Pro Asp
 565 570 575

Val Cys Asp Glu Phe Thr Gly Ile Glu Pro Leu Ser Glu Asp Ala Ile
 580 585 590

Ile Thr Gly Phe Arg Asn Val Thr Ile Cys Pro Asn Pro Glu Asp Thr
 595 600 605

Cys Asp Phe Ala Arg Ala Ala Arg Phe Val Ser Thr Pro Phe His Glu
 610 615 620

Ile Met Ser Leu Lys Asp Leu Pro Ser Asp Pro Glu Arg Leu Leu Pro
 625 630 635 640

Glu Glu Asp Leu Asp Val Lys Thr Ser Glu Asp Gln Gln Thr Ala Cys
 645 650 655

Gly Thr Ile Tyr Ser Gln Thr Leu Ser Ile Lys Lys Leu Ser Pro Ile
 660 665 670

Ile Glu Asp Ser Arg Glu Ala Thr His Ser Ser Gly Phe Ser Gly Ser
 675 680 685

Ser Ala Ser Val Ala Ser Thr Ser Ser Ile Lys Cys Leu Gln Ile Pro
 690 695 700

Glu Lys Leu Glu Leu Thr Asn Glu Thr Ser Glu Asn Pro Thr Gln Ser
 705 710 715 720

Pro Trp Cys Ser Gln Tyr Arg Arg Gln Leu Leu Lys Ser Leu Pro Glu
 725 730 735

Leu Ser Ala Ser Ala Glu Leu Cys Ile Glu Asp Arg Pro Met Pro Lys
 740 745 750

Leu Glu Ile Glu Lys Glu Ile Glu Leu Gly Asn Glu Asp Tyr Cys Ile
 755 760 765

Lys Arg Glu Tyr Leu Ile Cys Glu Asp Tyr Lys Leu Phe Trp Val Ala
 770 775 780

Pro Arg Asn Ser Ala Glu Leu Thr Val Ile Lys Val Ser Ser Gln Pro
 785 790 795 800

Val Pro Trp Asp Phe Tyr Ile Asn Leu Lys Leu Lys Glu Arg Leu Asn
 805 810 815

Glu Asp Phe Asp His Phe Cys Ser Cys Tyr Gln Tyr Gln Asp Gly Cys
 820 825 830

Ile Val Trp His Gln Tyr Ile Asn Cys Phe Thr Leu Gln Asp Leu Leu
 835 840 845

Gln His Ser Glu Tyr Ile Thr His Glu Ile Thr Val Leu Ile Ile Tyr
 850 855 860

Asn Leu Leu Thr Ile Val Glu Met Leu His Lys Ala Glu Ile Val His
 865 870 875 880

Gly Asp Leu Ser Pro Arg Cys Leu Ile Leu Arg Asn Arg Ile His Asp
 885 890 895

Pro Tyr Asp Cys Asn Lys Asn Asn Gln Ala Leu Lys Ile Val Asp Phe
 900 905 910

Ser Tyr Ser Val Asp Leu Arg Val Gln Leu Asp Val Phe Thr Leu Ser
 915 920 925

Gly Phe Arg Thr Val Gln Ile Leu Glu Gly Gln Lys Ile Leu Ala Asn
 930 935 940

Cys Ser Ser Pro Tyr Gln Val Asp Leu Phe Gly Ile Ala Asp Leu Ala
 945 950 955 960

His Leu Leu Leu Phe Lys Glu His Leu Gln Val Phe Trp Asp Gly Ser
 965 970 975

Phe Trp Lys Leu Ser Gln Asn Ile Ser Glu Leu Lys Asp Gly Glu Leu
 980 985 990

Trp Asn Lys Phe Phe Val Arg Ile Leu Asn Ala Asn Asp Glu Ala Thr
 995 1000 1005

Val Ser Val Leu Gly Glu Leu Ala Ala Glu Met Asn Gly Val Phe
 1010 1015 1020

Asp Thr Thr Phe Gln Ser His Leu Asn Lys Ala Leu Trp Lys Val
 1025 1030 1035

Gly Lys Leu Thr Ser Pro Gly Ala Leu Leu Phe Gln
 1040 1045 1050

<210> 13
 <211> 1085
 <212> PRT
 <213> Homo sapiens
 <400> 13

Met Asp Thr Pro Glu Asn Val Leu Gln Met Leu Glu Ala His Met Gln
 1 5 10 15

Ser Tyr Lys Gly Asn Asp Pro Leu Gly Glu Trp Glu Arg Tyr Ile Gln
 20 25 30

Trp Val Glu Glu Asn Phe Pro Glu Asn Lys Glu Tyr Leu Ile Thr Leu
 35 40 45

Leu Glu His Leu Met Lys Glu Phe Leu Asp Lys Lys Lys Tyr His Asn
 50 55 60

Asp Pro Arg Phe Ile Ser Tyr Cys Leu Lys Phe Ala Glu Tyr Asn Ser
 65 70 75 80

Asp Leu His Gln Phe Phe Glu Phe Leu Tyr Asn His Gly Ile Gly Thr
 85 90 95

Leu Ser Ser Pro Leu Tyr Ile Ala Trp Ala Gly His Leu Glu Ala Gln
 100 105 110

Gly Glu Leu Gln His Ala Ser Ala Val Leu Gln Arg Gly Ile Gln Asn
 115 120 125

Gln Ala Glu Pro Arg Glu Phe Leu Gln Gln Gln Tyr Arg Leu Phe Gln
 130 135 140

Thr Arg Leu Thr Glu Thr His Leu Pro Ala Gln Ala Arg Thr Ser Glu
 145 150 155 160

Pro Leu His Asn Val Gln Val Leu Asn Gln Met Ile Thr Ser Lys Ser
165 170 175

Asn Pro Gly Asn Asn Met Ala Cys Ile Ser Lys Asn Gln Gly Ser Glu
180 185 190

Leu Ser Gly Val Ile Ser Ser Ala Cys Asp Lys Glu Ser Asn Met Glu
195 200 205

Arg Arg Val Ile Thr Ile Ser Lys Ser Glu Tyr Ser Val His Ser Ser
210 215 220

Leu Ala Ser Lys Val Asp Val Glu Gln Val Val Met Tyr Cys Lys Glu
225 230 235 240

Lys Leu Ile Arg Gly Glu Ser Glu Phe Ser Phe Glu Glu Leu Arg Ala
245 250 255

Gln Lys Tyr Asn Gln Arg Arg Lys His Glu Gln Trp Val Asn Glu Asp
260 265 270

Arg His Tyr Met Lys Arg Lys Glu Ala Asn Ala Phe Glu Glu Gln Leu
275 280 285

Leu Lys Gln Lys Met Asp Glu Leu His Lys Lys Leu His Gln Val Val
290 295 300

Glu Thr Ser His Glu Asp Leu Pro Ala Ser Gln Glu Arg Ser Glu Val
305 310 315 320

Asn Pro Ala Arg Met Gly Pro Ser Val Gly Ser Gln Gln Glu Leu Arg
325 330 335

Ala Pro Cys Leu Pro Val Thr Tyr Gln Gln Thr Pro Val Asn Met Glu
340 345 350

Lys Asn Pro Arg Glu Ala Pro Pro Val Val Pro Pro Leu Ala Asn Ala
355 360 365

Ile Ser Ala Ala Leu Val Ser Pro Ala Thr Ser Gln Ser Ile Ala Pro
370 375 380

Pro Val Pro Leu Lys Ala Gln Thr Val Thr Asp Ser Met Phe Ala Val
385 390 395 400

Ala Ser Lys Asp Ala Gly Cys Val Asn Lys Ser Thr His Glu Phe Lys

24

Ile Gln Glu Lys Ser Pro Lys Gln Ala Leu Ser Ser His Met Tyr Ser
 660 665 670

Ala Ser Leu Leu Arg Leu Ser Gln Pro Ala Ala Gly Gly Val Leu Thr
 675 680 685

Cys Glu Ala Glu Leu Gly Val Glu Ala Cys Arg Leu Thr Asp Thr Asp
 690 695 700

Ala Ala Ile Ala Glu Asp Pro Pro Asp Ala Ile Ala Gly Leu Gln Ala
 705 710 715 720

Glu Trp Met Gln Met Ser Ser Leu Gly Thr Val Asp Ala Pro Asn Phe
 725 730 735

Ile Val Gly Asn Pro Trp Asp Asp Lys Leu Ile Phe Lys Leu Leu Ser
 740 745 750

Gly Leu Ser Lys Pro Val Ser Ser Tyr Pro Asn Thr Phe Glu Trp Gln
 755 760 765

Cys Lys Leu Pro Ala Ile Lys Pro Lys Thr Glu Phe Gln Leu Gly Ser
 770 775 780

Lys Leu Val Tyr Val His His Leu Leu Gly Glu Gly Ala Phe Ala Gln
 785 790 795 800

Val Tyr Glu Ala Thr Gln Gly Asp Leu Asn Asp Ala Lys Asn Lys Gln
 805 810 815

Lys Phe Val Leu Lys Val Gln Lys Pro Ala Asn Pro Trp Glu Phe Tyr
 820 825 830

Ile Gly Thr Gln Leu Met Glu Arg Leu Lys Pro Ser Met Gln His Met
 835 840 845

Phe Met Lys Phe Tyr Ser Ala His Leu Phe Gln Asn Gly Ser Val Leu
 850 855 860

Val Gly Glu Leu Tyr Ser Tyr Gly Thr Leu Leu Asn Ala Ile Asn Leu
 865 870 875 880

Tyr Lys Asn Thr Pro Glu Lys Val Met Pro Gln Gly Leu Val Ile Ser
 885 890 895

Phe Ala Met Arg Met Leu Tyr Met Ile Glu Gln Val His Asp Cys Glu

900	905	910
Ile Ile His Gly Asp Ile Lys Pro Asp Asn Phe Ile Leu Gly Asn Gly 915 920 925		
Phe Leu Glu Gln Asp Asp Glu Asp Asp Leu Ser Ala Gly Leu Ala Leu 930 935 940		
Ile Asp Leu Gly Gln Ser Ile Asp Met Lys Leu Phe Pro Lys Gly Thr 945 950 955 960		
Ile Phe Thr Ala Lys Cys Glu Thr Ser Gly Phe Gln Cys Val Glu Met 965 970 975		
Leu Ser Asn Lys Pro Trp Asn Tyr Gln Ile Asp Tyr Phe Gly Val Ala 980 985 990		
Ala Thr Val Tyr Cys Met Leu Phe Gly Thr Tyr Met Lys Val Lys Asn 995 1000 1005		
Glu Gly Gly Glu Cys Lys Pro Glu Gly Leu Phe Arg Arg Leu Pro 1010 1015 1020		
His Leu Asp Met Trp Asn Glu Phe Phe His Val Met Leu Asn Ile 1025 1030 1035		
Pro Asp Cys His His Leu Pro Ser Leu Asp Leu Leu Arg Gln Lys 1040 1045 1050		
Leu Lys Lys Val Phe Gln Gln His Tyr Thr Asn Lys Ile Arg Ala 1055 1060 1065		
Leu Arg Asn Arg Leu Ile Val Leu Leu Leu Glu Cys Lys Arg Ser 1070 1075 1080		
Arg Lys 1085		
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Glu Phe Pro Glu Val Phe Met Met Glu Val Lys Asp Pro Asn Met Lys
 20 25 30

Gly Ala Met Leu Thr Asn Thr Gly Lys Tyr Ala Ile Pro Thr Ile Asp
 35 40 45

Ala Glu Ala Tyr Ala Ile Gly Lys Lys Glu Lys Pro Pro Phe Leu Pro
 50 55 60

Glu Glu Pro Ser Ser Ser Ser Glu Glu Asp Asp Pro Ile Pro Asp Glu
 65 70 75 80

Leu Leu Cys Leu Ile Cys Lys Asp Ile Met Thr Asp Ala Val Val Ile
 85 90 95

Pro Cys Cys Gly Asn Ser Tyr Cys Asp Glu Cys Ile Arg Thr Ala Leu
 100 105 110

Leu Glu Ser Asp Glu His Thr Cys Pro Thr Cys His Gln Asn Asp Val
 115 120 125

Ser Pro Asp Ala Leu Ile Ala Asn Lys Phe Leu Arg Gln Ala Val Asn
 130 135 140

Asn Phe Lys Asn Glu Thr Gly Tyr Thr Lys Arg Leu Arg Lys Gln Leu
 145 150 155 160

Pro Pro Pro Pro Pro Pro Ile Pro Pro Pro Arg Pro Leu Ile Gln Arg
 165 170 175

Asn Leu Gln Pro Leu Met Arg Ser Pro Ile Ser Arg Gln Gln Asp Pro
 180 185 190

Leu Met Ile Pro Val Thr Ser Ser Ser Thr His Pro Ala Pro Ser Ile
 195 200 205

Ser Ser Leu Thr Ser Asn Gln Ser Ser Leu Ala Pro Pro Val Ser Gly
 210 215 220

Asn Pro Ser Ser Ala Pro Ala Pro Val Pro Asp Ile Thr Ala Thr Val
 225 230 235 240

Ser Ile Ser Val His Ser Glu Lys Ser Asp Gly Pro Phe Arg Asp Ser
 245 250 255

Asp Asn Lys Ile Leu Pro Ala Ala Ala Leu Ala Ser Glu His Ser Lys
 260 265 270

Gly Thr Ser Ser Ile Ala Ile Thr Ala Leu Met Glu Glu Lys Gly Tyr
 275 280 285

Gln Val Pro Val Leu Gly Thr Pro Ser Leu Leu Gly Gln Ser Leu Leu
 290 295 300

His Gly Gln Leu Ile Pro Thr Thr Gly Pro Val Arg Ile Asn Thr Ala
 305 310 315 320

Arg Pro Gly Gly Gly Arg Pro Gly Trp Glu His Ser Asn Lys Leu Gly
 325 330 335

Tyr Leu Val Ser Pro Pro Gln Gln Ile Arg Arg Gly Glu Arg Ser Cys
 340 345 350

Tyr Arg Ser Ile Asn Arg Gly Arg His His Ser Glu Arg Ser Gln Arg
 355 360 365

Thr Gln Gly Pro Ser Leu Pro Ala Thr Pro Val Phe Val Pro Val Pro
 370 375 380

Pro Pro Pro Leu Tyr Pro Pro Pro Pro His Thr Leu Pro Leu Pro Pro
 385 390 395 400

Gly Val Pro Pro Pro Gln Phe Ser Pro Gln Phe Pro Pro Gly Gln Pro
 405 410 415

Pro Pro Ala Gly Tyr Ser Val Pro Pro Gly Phe Pro Pro Ala Pro
 420 425 430

Ala Asn Leu Ser Thr Pro Trp Val Ser Ser Gly Val Gln Thr Ala His
 435 440 445

Ser Asn Thr Ile Pro Thr Thr Gln Ala Pro Pro Leu Ser Arg Glu Glu
 450 455 460

Phe Tyr Arg Glu Gln Arg Arg Leu Lys Glu Glu Glu Lys Lys Lys Ser
 465 470 475 480

Lys Leu Asp Glu Phe Thr Asn Asp Phe Ala Lys Glu Leu Met Glu Tyr
 485 490 495

Lys Lys Ile Gln Lys Glu Arg Arg Arg Ser Phe Ser Arg Ser Lys Ser
 500 505 510

Pro Tyr Ser Gly Ser Ser Tyr Ser Arg Ser Ser Tyr Thr Tyr Ser Lys
515 520 525

Ser Arg Ser Gly Ser Thr Arg Ser Arg Ser Tyr Ser Arg Ser Phe Ser
530 535 540

Arg Ser His Ser Arg Ser Tyr Ser Arg Ser Pro Pro Tyr Pro Arg Arg
545 550 555 560

Gly Arg Gly Lys Ser Arg Asn Tyr Arg Ser Arg Ser Arg Ser His Gly
565 570 575

Tyr His Arg Ser Arg Ser Arg Ser Pro Pro Tyr Arg Arg Tyr His Ser
580 585 590

Arg Ser Arg Ser Pro Gln Ala Phe Arg Gly Gln Ser Pro Asn Lys Arg
595 600 605

Asn Val Pro Gln Gly Glu Thr Glu Arg Glu Tyr Phe Asn Arg Tyr Arg
610 615 620

Glu Val Pro Pro Pro Tyr Asp Met Lys Ala Tyr Tyr Gly Arg Ser Val
625 630 635 640

Asp Phe Arg Asp Pro Phe Glu Lys Glu Arg Tyr Arg Glu Trp Glu Arg
645 650 655

Lys Tyr Arg Glu Trp Tyr Glu Lys Tyr Tyr Lys Gly Tyr Ala Ala Gly
660 665 670

Ala Gln Pro Arg Pro Ser Ala Asn Arg Glu Asn Phe Ser Pro Glu Arg
675 680 685

Phe Leu Pro Leu Asn Ile Arg Asn Ser Pro Phe Thr Arg Gly Arg Arg
690 695 700

Glu Asp Tyr Val Gly Gly Gln Ser His Arg Ser Arg Asn Ile Gly Ser
705 710 715 720

Asn Tyr Pro Glu Lys Leu Ser Ala Arg Asp Gly His Asn Gln Lys Asp
725 730 735

Asn Thr Lys Ser Lys Glu Lys Glu Ser Glu Asn Ala Pro Gly Asp Gly
740 745 750

Lys Gly Asn Lys His Lys Lys His Arg Lys Arg Arg Lys Gly Glu Glu
755 760 765

Ser Glu Gly Phe Leu Asn Pro Glu Leu Leu Glu Thr Ser Arg Lys Ser
 770 775 780

Arg Glu Pro Thr Gly Val Glu Glu Asn Lys Thr Asp Ser Leu Phe Val
 785 790 795 800

Leu Pro Ser Arg Asp Asp Ala Thr Pro Val Arg Asp Glu Pro Met Asp
 805 810 815

Ala Glu Ser Ile Thr Phe Lys Ser Val Ser Glu Lys Asp Lys Arg Glu
 820 825 830

Arg Asp Lys Pro Lys Ala Lys Gly Asp Lys Thr Lys Arg Lys Asn Asp
 835 840 845

Gly Ser Ala Val Ser Lys Lys Glu Asn Ile Val Lys Pro Ala Lys Gly
 850 855 860

Pro Gln Glu Lys Val Asp Gly Asp Val Arg Asp Leu Leu Asp Leu Asn
 865 870 875 880

Leu Gln Leu Lys Lys Pro Lys Arg Arg Leu Arg Arg Leu Thr Ile Leu
 885 890 895

Asn His His Leu Pro Leu Arg Arg Met Lys Lys Ser Leu Glu Pro Pro
 900 905 910

Glu Lys Leu Thr Leu Asn Gln Gln Lys Thr Pro Arg Asn Lys Thr Ser
 915 920 925

Gln Arg Gly Lys Ser Glu Glu Gly Leu Phe Gln Arg Cys Gln Ile Arg
 930 935 940

Lys Ala Asn Asn
 945